Cell Cultures for Virus Vaccine Production

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CONFERENCE
ON
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Conference Chairman
and Editor
Donald J. Merchant

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PREFACE

This Conference was called to provide a forum for discussion of the properties of cell culture systems as they are related to virus vaccine production. It is fortunate that developments over the past three decades have provided us with at least three distinct kinds of cell culture systems, each of which has the capability of mass production of a wide range of virus vaccines. These are 1) primary cultures, 2) diploid or finite cell lines, and 3) continuous cell lines.

Each of these systems has its own peculiar characteristics which influence its suitability for production of virus vaccines. Some properties such as nutritional requirements, ease of handling, rate of growth, range of virus susceptibility, and level of virus yield have great practical significance. Others such as chromosome composition, loss of specific function, and altered metabolic patterns are more difficult to evaluate. Possible tumorigenicity of cells and the presence of latent viruses or of oncogenic agents are additional complicating factors of great significance.

It was the hope of the Division of Biologics Standards and of the Conference organizing committee that these and other facets of the problem before us might be discussed openly and candidly so that a critical evaluation of the current status could be made. While it was not realistic to expect such a large and diversified group to come to agreement on any major segments of the topic, it was felt that an important service could be rendered by presenting all the material concisely, objectively, and without bias.

It was of the greatest importance that all participants should keep within their allotted time and address themselves pointedly to the assigned topics. We tried to ensure ample time for free discussion so that we might have the full benefit of pertinent comments from visitors as well as participants. Additional time for review of all the facets of this program was available on Wednesday afternoon in the final discussion period.

I would like to express my gratitude to all the participants for the willingness and enthusiasm with which they accepted our invitation and for their cooperation throughout the planning stages. I also would like to thank the members of the planning committee and those persons who participated in the preconference session on terminology for their invaluable help. It has been a particular pleasure to work with Dr. Murray and his staff at the Division of Biologics Standards.

Donald J. Merchant

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The program arranged for this Conference was very extensive and varied. No conference, of course, can be completely comprehensive, so it would have been naive for us to expect answers to all of the various problems presented.

However, such a great deal of work had been done since the January 4, 1963, report which appeared in *Science* on the use of continuous cell lines for virus production that it was appropriate to review both published and unpublished work at this time. As a matter of fact, I might say that it was a little overdue.

It was hoped that this Conference would provide a record that could be used as a base from which it might be possible to proceed to the consideration of policies relative to the use of primary and continuous cell lines for the production of virus vaccines.

I believe that perhaps the most important dividend and feature of this Conference was the opportunity for the open discussion at the end of each session. I think that as a result of its vigor and liveliness, a great deal of benefit accrued to all.

RODERICK MURRAY

SESSION I

CHARACTERISTICS OF CELL CULTURE SYSTEMS

Chairman: VIRGINIA J. EVANS
Rapporteur: JOHN H. HANKS

Phenotypic Expression and Cell Marker Systems[1]

Morgan Harris, *Department of Zoology, University of California, Berkeley, California 94720*

THIS meeting begins appropriately with a discussion of primary cultures and diploid cells, since these were the materials that Alexis Carrel and his fellow pioneers used in a first search for pure lines within explant systems. In historical perspective, these efforts were not notably successful, and interest in freshly isolated cells declined, especially when it was shown about 1950 that strain L, HeLa, and other established lines could readily be cultivated in monolayer and suspension forms. In recent years, the pendulum has swung back again, and we now recognize the experimental usefulness of primary diploid cells as well as heteroploid lines, among other reasons for the purpose of virus vaccine production to which this Conference is devoted. Our special charge at this time is the properties of freshly isolated cell populations, with special reference to factors that affect their characterization *in vitro*. For practical purposes, we need reliable fingerprints that can serve as identification standards, enabling us to differentiate cells that are derived from substrains within a species as well as from different species. In addition, we seek criteria that will go further: markers that will document the origin of cell types in culture from precursor tissues and organs of the intact animal.

However, there is a third and much more important factor which I want to stress particularly. This is the phenomenon of modulation, first characterized by Paul Weiss (*1*) many years ago. According to this concept, differentiation of somatic cells is essentially irreversible, once morphogenetic determination has occurred. On the other hand, the manifestations of differentiation are more variable and can be strongly influenced by environmental factors. For example, pigment granules or other specialized products can disappear from freshly isolated cells under given conditions of culture. But the same markers may reappear if the environment is altered appropriately, showing that no permanent change has occurred

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[1] Supported in part by Public Health Service grant GM 13692 from the National Institute of General Medical Sciences.

within the cells concerned. Thus, modulation refers to reversible shifts in phenotypic expression that take place against an unchanging background of genetic information. Unfortunately, the mechanisms of modulation are still obscure to us. We do not know whether reversibility implies repression and derepression of genes directly concerned, or whether the key to modulation lies at an epigenetic level. But in any event, the significance of modulation in the present context is clear: Morphogenetic shifts of this must obviously be considered in any discussion of cell markers *in vitro.*

STABILITY OF CELLULAR PHENOTYPES

To give meaning to these general remarks, we should consider the expression of particular characteristics within the cells of primary cultures and derivative diploid lines. One finds a wide spectrum of differences in the stability of individual markers and corresponding differentials in potential for modulative change.

Differentiation of cartilage.—Dissociated populations of cartilage cells are readily obtained from embryonic chick rudiments, and they display a distinctive pattern of specialized markers when first explanted *in vitro.* As Holtzer and his colleagues have shown (*2–4*), these features include a distinctive morphology, selective incorporation of ^{35}S into chondroitin sulfate, and elaboration of a mucopolysaccharide matrix that can be recognized by metachromatic staining. In Holtzer's experiments, however, chondrogenesis did not appear to be a stable phenomenon in the cell populations concerned. For example, when chick cartilage cells were cultivated as monolayers in a medium made up of serum and embryo extract, the cells gradually lost their distinctive properties. After serial transfer, these populations assumed a fibroblast-like appearance, and no longer showed matrix formation or significant uptake of ^{35}S. Holtzer was unable to reverse this shift experimentally.

Newer information, however, makes it clear that changes in cultures of cartilage cells are based on modulation rather than progressive dedifferentiation. The data of Coon (*5*, *6*) are particularly noteworthy. Coon examined the effects of cell density and growth phase on chrondrogenesis in cultures of chick cartilage cells, and the embryo extract used was fractionated on Sephadex columns. Surprisingly, the high molecular weight fraction appeared to inhibit cartilage formation. By using a medium in which this component was excluded, Coon obtained continuous formation of cartilage by clonal populations for 35 cell generations or more. Cell density also proved to be a parameter of modulative change. Coon found that, if chondroblasts were grown as mass populations in monolayers, expression of specialized function ceased, as Holtzer had previously observed. But when such cells were dissociated and inoculated in small numbers to form clonal subcultures, chondrogenesis reappeared in unmistakable fashion. Thus, it is apparent that the potential for cartilage formation can disappear and reappear as reversible expression of phenotype, and without loss of basic differentiation in the cells concerned.

Transplantation antigens.—Quite in contrast to the system we have been discussing are the cellular markers responsible for histocompatibility in mammalian cells. An extensive literature (*7*) now shows that the individuality of these antigens is specified by genetic factors, whether one is dealing with differences that separate species, or with isoantigenic differentials which may exist between inbred strains. All evidence points to an extraordinary stability of transplantation antigens in terms of phenotypic expression. No significant modulation in the occurrence or strength of these antigens has been observed in primary cultures or diploid cell lines, nor have mutations at histocompatibility loci been reliably reported. Within established lines, somewhat more variability in isoantigenic pattern has been observed, as in tumors. Qualitative as well as quantitative variants at the H-2 locus in mouse cells have now been isolated (*8*, *9*). No similar occurrences, however, have been observed within unmodified diploid cells. And even among established cell types, the persistence of antigenic specificity is remarkable. Thus, Cann and Herzenberg (*10*) showed that stock populations of ML388 mouse lymphoma cells, even after 6 years in heterologous media, still retained the H-2b isoantigenic phenotype. In more comprehensive studies, Gangal and associates (*11*) found that strain L cells possessed all the specificities characteristic of H-2k in C3H mice, from which this line had been derived some 25 years previously. These results were extended further by the work of Drysdale and co-workers (*12*) who demonstrated that constancy in H-2 specificities is maintained regardless of variations that may take place in karyotype or expression of alkaline phosphatase activity within the cells concerned.

Tissue-specific and blood group antigens.—Lest one might conclude that antigens as a class are immutable, we should note that tissue- and organ-specific antigens are highly unstable *in vitro;* typically, these disappear soon after explantation (*13*). The experiments of Weiler (*14*) provide a good illustration. Weiler prepared fresh cultures of hamster kidney epithelium, in which he observed initially a binding of kidney-specific antibody, with lysis in the presence of complement. After incubation for 3 days, however, cytotoxicity could not be observed, and selective binding of antibody by epithelial cells did not take place. However, these populations showed no diminution in ability to bind species-specific antibody during the same period. Other authors have described a similar reduction or disappearance of tissue-specific antigens in cultures of thyroid, colon, and liver (*13*). Up to the present time, at least, re-expression of tissue-specific antigens by modulation has not been demonstrated in these systems, but it is reasonable to envision this possibility by suitable manipulation of cultural conditions.

Studies on blood group antigens, in particular A and B, reveal additional degrees of freedom in the expression of cell markers. As Coombs and his collaborators (*15*) showed in 1956, antigens A and B can be demonstrated in freshly isolated human tissue cells but are not detectable after prolonged cultivation (*16*, *17*). On the other hand, A and B antigens in rabbit cells persist for long periods *in vitro.* They show, however, a conspicuous potential for intercellular variation (*18*). More specifically, clones derived from the RK13 epithelial line were found to consist of mixtures of antigen-positive and antigen-negative cells. The latter were not loss variants, for on isolation they could give rise again to cells in which antigen synthesis occurred. It is not yet clear how the switch in production of antigens is achieved, but the high frequency of change is not in accordance with a mutation hypothesis. A more reasonable interpretation is that of modulation, based on local changes in the microenvironment within single colonies.

Forssman-type antigens.—Although modulation refers ordinarily to cell characters in being, it can be extended to include the induction and repression of markers which exist only in potential form. The experiments of Fogel and Sachs (*19*, *20*) illustrate this phenomenon. Cultures were prepared in monolayer form from hamster and mouse tissues, and examined by immunofluorescence for Forssman antigen. In preparations incubated for 1 day, heterophile antigen of the Forssman type could not be detected. After 3 days, however, nearly all cells in monolayer populations were positive. The kinetics of this process suggest that Forssman-type antigen appears through an inductive process and is not the result of selective overgrowth by a minority cell type. Although the nature of induction is not clear, it seems correlated with creation of free cell surfaces in monolayers, for production of Forssman-type antigens in these cells can again be repressed by aggregating the cells and culturing in pellet form. It is possible, therefore, that the production of heterophile antigens is initiated by the disruption of normal cell contacts that exist *in vivo;* this process ceases when an association is restored by experimental means.

DIFFERENTIAL DIAGNOSIS OF CELL TYPES

In considering the utility of cell markers for practical problems of recognition, the take-home lesson is that standards currently available are of necessity based on characters with a constant phenotypic expression. This list could be expanded to take advantage of more variable markers if, as in the case of cartilage formation, means could be found to stabilize the effects of modulative change. The need is particularly pressing for identification of cell types within diploid lines from such organs as liver or kidney. Here, histological and morphogenetic distinctions exist in the precursor populations. The problem is to preserve and refine these distinctions to a more usable level *in vitro.*

Turning from potential identification standards to those that are now available, obviously we have a considerable and growing array of experimental tools. A partial listing would include the categories mentioned briefly below; others will be mentioned in subsequent discussion.

Chromosomal patterns.—The usefulness of karyotypic analysis for species identification scarcely requires comment and is of optimal value in the characterization of unmodified diploid cells (*21*, *22*). Intraspecific recognition by chromosome pattern would also become possible if unique marker chromosomes could be introduced by experimental treatment, *e.g.*, by X irradiation, as accomplished by Ruddle (*23*) with an established line of pig kidney cells. Unfortunately this procedure and the incorporation of other useful markers such as drug resistance are not presently feasible for diploid cell lines. The stumbling block is the number of cell generations required to isolate such variants in clonal form from a parental population. This would consume a large fraction of the viable proliferation span for human diploid lines, or of the period prior to heteroploid transformation in other cell types (*24*, *25*).

As an alternative means for intraspecific recognition, sex chromatin or sex chromosomes, where morphological differences are clear-cut, offer useful markers. They have been used, for example, to assess the degree of secondary tumor induction by virus in transplantations of Rous sarcoma cells (*26*). The T–6 translocation introduced into CBA mice is also well known as a marker. Originating by X irradiation in the genetic studies of Carter, Lyon, and Phillips (*27*), the T–6 chromosome has provided an identifying tag for numerous studies, *e.g.*, cytological identification of donor and host cells in radiation chimeras (*28*) and verification of cell fusion in hybridization experiments (*29*).

Serological diagnosis.—The recognition of species origin has been solved satisfactorily for isolated cell populations by several immunochemical procedures (*30*). The early studies of Brand and Syverton (*31*, *32*) with hemagglutination showed that consistent differences in antigenic pattern are to be found for established cell lines as well as primary cultures that correspond to different species. Equally clear-cut species distinctions may be made by mixed agglutination (*33*, *34*) or immunofluorescence procedures (*35*, *36*). As noted earlier, isoantigenic specificity at the H-2 locus in mouse cells permits serological diagnosis of diploid or heteroploid lines if these have contrasting alleles. In principle, similar differences at histocompatibility loci would permit a broad pattern of intraspecific distinctions among animal cells. However, the usefulness of these techniques is limited by the lack of inbred stocks for most species of animals, and by serological reactions that are not clear-cut except for strong histocompatibility factors such as the H-2 locus of the mouse.

Enzymatic characterizations.—A number of stable markers for intraspecific studies have been obtained by isolating cells from individuals with heritable biochemical deficiencies. The list cited by Gartler and Pious (*37*) includes acatalasia, galactosemia, Hurler's disease, orotic aciduria, and maple sugar disease. In addition, individuals may be found with genetic variations in several specific enzymes: glucose-6-phosphate dehydrogenase, phosphoglucomutase, 6-phosphogluconic dehydrogenase, and lactic acid dehydrogenase. These markers are detectable at the cellular level and for the most part are not significantly influenced by phenotypic modulation. They have the necessary specificity to serve as identification standards. Comment in detail on these systems and on other studies of enzymatic patterns will be provided by our discussants. This is a rapidly expanding field and one which holds substantial promise for our immediate goal, the functional characterization of diploid cell lines.

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Isozymic Variants as Genetic Markers in Somatic Cell Populations *In Vitro*

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I would like to focus attention on the use of isozymic markers for the genetic analysis of somatic cell populations. Isozymes are enzymes that possess a single catalytic specificity but which differ in their electrophoretic mobilities. Certain properties of isozymes commend them as somatic cell markers: 1) Isozymic patterns are generally highly distinctive between different species; 2) genetically determined isozymic variants occur at a relatively high frequency within species, as demonstrated by Harris (*1*) for man, Lewontin and Hubby (*2*) for *Drosophila*, and by Roderick *et al.* (*3*) for the mouse *Mus musculus;* 3) the genetic control of isozyme variants is generally simple, and in many instances the genes are codominant. Thus, the isozyme genotypes can be deduced explicitly and precisely from the phenotypes; and 4) since most enzymes are polymorphic and since many enzymes exist, then the isozymes represent a potentially very large class of markers. These points are now relatively well established.

I would like to demonstrate the following two additional points: 1) Isozymes are sufficiently stable *in vitro* to be used as genetic markers, and 2) isozyme markers in heterozygous configurations can provide information on genetic alterations in evolving *in vitro* cell populations.

We have examined recently isozyme variants in the mouse for the enzymes malate dehydrogenase (*4*) (MDH), isocitrate dehydrogenase (*5*) (IDH), glucose-6-phosphate dehydrogenase (*6*) (G6PD), and phosphoglucomutase (PGM). MDH, IDH, and G6PD each possess 2 variant alleles at their separate respective loci. It has been ascertained that the alleles are expressed codominantly and that they are not closely linked. The PGM variants have been detected only recently, but our preliminary studies indicate that PGM behaves genetically like the other enzyme markers.

By surveying the inbred strains of mice, we have found 2 strains which carry different variant forms for these polymorphic enzymes. These strains are SJL/J and C57BL/6J. Hybrid F_1 animals between SJL/J and C57BL/6J express unique hybrid patterns which differ from the parental.

Tissue culture cell populations have been prepared from fetuses of the parental and hybrid types. All 3 populations are fibroblastic and heteroploid after more than 1 year in continuous culture. It would be extremely difficult to ascertain the origins of these 3 lines by their growth properties, morphologies, and/or karyotypes. However, such discrimination and identification can be accomplished readily by isozyme analysis. A cell population can be recognized unequivocally as to strain of origin by examination of its isozyme pattern. In these experiments, 4 loci each possessed 2 possible alleles. This generated 16 recognizable isozymic patterns. If the number of loci varying for 2 alleles were increased to 10, the number of recognizable classes would have increased to 1,000. These considerations suggest the potential discriminatory power of this system.

These results demonstrate that isozyme markers are stably expressed in heteroploid cultures after cultivation for more than 1 year. Other studies on 11 established murine tissue culture populations derived from animals of the C3H, BALB, and DBA strains express—even after 10 years of cultivation in some instances (*7*)—isozyme marker patterns consistent with the respective strains of origin.

The isozyme patterns of the heterozygous (C57 × SJL)F_1 cell population have provided intriguing data (fig. 1). MDH and PGM heterozygote patterns are consistent with a balanced diploid condition for those loci. The G6PD pattern is consistent with the loss or repression of the SJL variant allele. The IDH pattern can be explained by assuming the addition of one additional SJL variant allele. A trisomic condition would be expected to yield a ratio of isozyme intensity of 4 : 4 : 1. The zymogram for the IDH hybrid does not significantly depart from such an expectation. The heteroploid line can be presumed, therefore, to be disomic at the PGM and MDH loci, monosomic at the G6PD locus, and trisomic at the IDH locus. It has been presumed for many years that heteroploidy represents a genetically unbalanced condition. These results provide direct evidence supporting that view. More significantly, the system illustrates a method by which genetic transitions can be traced in somatic cell populations for multiple markers with great sensitivity.

Isozyme variants thus represent a versatile and powerful class of genetic markers especially suited to the requirements of *in vitro* somatic cell genetics. The data I have presented suggest strategies for the solution of problems posed by this meeting. During our discussions, we can inquire into these possibilities.

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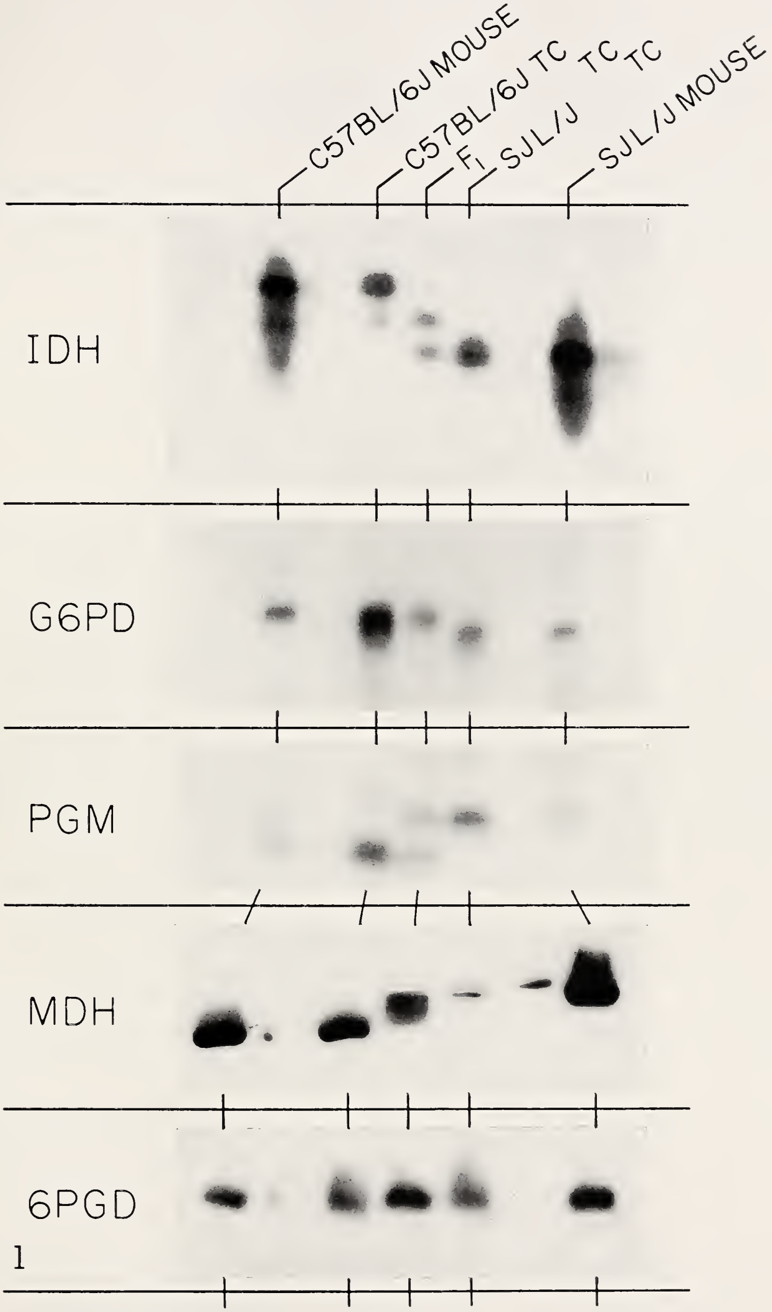
C57BL/6J MOUSE
C57BL/6J TC
F1 TC
SJL/J TC
SJL/J MOUSE
IDH
G6PD
PGM
MDH
6PGD
1

FIGURE 1.—Isozyme patterns of tissues from 2 mouse strains, from a tissue culture derived from each strain, and from a tissue culture derived from an F_1 hybrid of the 2 strains. Genetic variation has not been described for 6PGD in the mouse.

RUDDLE

Identification Standards of Primary Cultures and Diploid Cell Lines—Discussion

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One aspect of Dr. Harris's remarks has been of particular concern to those of us who are responsible for the practical identification of cells—especially in the preparation of reference cell cultures. Whether one is dealing with primary and diploid cells of limited or finite lifespans, *or* with established lines, we need to be able to identify them. We are not referring to modulations or variations, both of which can include virus susceptibility. We have to identify cells genetically. We can do so in two ways, either between species (*1*) or within a species (*2*, *3*). In some, but not all instances, identification might be accomplished karyologically. As a matter of fact, this was the first way in which it was demonstrated that a line presumed to be of a certain species was shown to be actually of another species origin. Such interspecies contaminations led us and others (*4*) to work out serologic methods that would put species identifications of cells on a practical basis. In recent years it has become routine to identify any cell line with regard to species of origin, and these days there is no reason for anyone to be dealing with cells in which the species is unknown or has been contaminated with cells of other species. In a simple method worked out in our laboratories we use specific fluorescent-labeled antisera with living cells, resulting in peripheral fluorescence of a specific reaction. This is a stable character which is specified by genetic factors.

Throughout the years we have found many examples wherein investigators have unwittingly been working with cells of diverse species origins (*1*, *4*). If such is the case it would be unreasonable to expect that this also would not occur *within* a given species. Until recently there have not been reliable genetic markers to identify intraspecies characters. However, the utilization of isoenzyme polymorphisms as genetic intraspecies identification markers has now become a real possibility (*2*). As shown by Ruddle, as well as in discussions later in this Conference, it is becoming possible to use such isoenzymes to unmistakably identify any population of cells in culture.

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GENERAL DISCUSSION

Dr. I. S. Johnson (Lilly Research Laboratories) : Dr. Ruddle, it wasn't clear to me whether the future culture lines were primary cultures. Did you try any hybridization? Were the cells *in vitro* diploid or heteroploid? Also, have you tried somatic hybridization experiments?

Dr. F. Ruddle: The populations that I have just reported on were cultured from mouse fetuses of the appropriate genetic stocks. The lines have been in cultivation for over 1 year—approaching 18 months. The populations are heteroploid, showing chromosome numbers of 50–60. Actually, the chromosome configurations, although heteroploid and all murine, do show some differences between the individual lines.

With regard to somatic cell hybridization, we are carrying out studies of this sort. We have just recently had an opportunity to look at the HM–2 cell population which was initiated by Mary Weiss in a hybridization cross, involving cell fusion between LM, TK–, a strain of murine origin, and WI–38, a strain of human origin.

This hybrid population has evolved considerably, so that practically all chromosomes now are of the murine type. In our studies of this hybrid cell population for MDH, IDH, G6PD, and PGM, we find that only the murine enzymes are expressed in that cell population. We find, however, that for LDH there is a hybrid enzyme which can be explained best as the hybrid product of subunits of murine and human origin. So that it appears that the human allele for the LDH–5 subunit still resides in the hybrid line.

We have not been able to ascertain what human chromosome is correlated with the LDH pattern. But, of course, we are interested in doing so.

Dr. J. Mills (National Institute of Allergy and Infectious Diseases) : Dr. Stulberg, have you evidence for the specificity of this fluorescent antibody reaction? How small a difference will it show up?

Dr. C. S. Stulberg: It will show differences between species unmistakably. This has been done hundreds and hundreds of times by all those who are interested in that aspect. Whether it shows differences within a species, it's hard to say.

Differences that may be apparent may be due to different concentrations of antisera or perhaps to methods in which cells were prepared. You can see differences, for example, between heteroploid and diploid cells. Of course, you don't need fluorescent antibodies to see these. But these differences, the small differences, are hard to identify and to determine just exactly what they mean. It is possible to detect one in 5,000 cells in species mixtures, however.

But between species there is no question. It is an "all or none" phenomenon and can be done rather easily not only by this means but by other means, as you know.

Dr. D. J. Merchant: In the case of the mouse cells and histocompatibility antigens, of course, due to the stability that is similar to the stability of species antigen, one has the same precision, and one can recognize a cell from a C3H mouse and a strain from a Swiss mouse or another mouse of another H–2 phenotype very readily, and one can detect one or two contaminating cells in a large population. It is a very sensitive method.

Dr. R. E. Stevenson: In 1966 at the Bedford Springs tissue culture conference, Dr. Gartler presented information on the G6PD isozyme background of an established cell line. Has this work been carried further so that any determinations have been made now as to whether these cell lines are indeed from the same source or are different? I throw that open to the group for discussion.

Dr. L. Coriell: I might respond to that for the cooperating laboratories of the Cell Cuture Collection.

Since last year, three laboratories have confirmed Dr. Gartler's report that G6PD isoenzyme variant type A is present in virtually every heteroploid human cell line that we all use in the laboratory. Whether this means that many of these cells have been contaminated by HeLa I don't know. HeLa does have the abnormal isoenzyme, and it is present only in a small percentage of colored people. To confirm the suspicion that many human cell lines are actually HeLa variants will require more work with other enzymes that Dr. Ruddle described earlier. But on the basis of G6PD studies alone, as I will show later in my paper at this Conference, most of the heteroploid human cell lines resemble HeLa.

Dr. G. Yerganian: If there is no further discussion on Dr. Gartler's work, could Dr. Harris make some comments regarding the applicability of somatic fusions for viral production?

Dr. M. Harris: What aspect of this did you have in mind?

Dr. Yerganian: As a cell source.

Dr. Harris: There is, of course, the possibility of creating *de novo* a great variety of cell types, and of recombining specific makers in new combinations.

Dr. K. K. Sanford: I want to make one comment on the effort to set up "identification standards" for primary cultures. Primary cultures usually are such heterogeneous populations of cells that the identification markers probably will have to be properties common to many different cell types, such as chromosome number or species or strain specificities. For instance, if an embyro is taken, or even a differentiated tissue of an adult, so many different cell types may survive and proliferate in primary culture that rigorous characterization of the cell populations would be difficult.

Dr. S. J. Pirt: Dr. Ruddle, do you find very marked variations in the amounts of any of these particular enzymes which you are actually using as markers? Can this in fact vitiate the method as a means of identifying cells?

Dr. Ruddle: That is an important consideration and as yet we have not had sufficient experience for me to answer it in any depth. However, with the system that I have described, the amount of enzyme seems to be related to the genetic dosage of a particular allele.

Dr. Pirt: Have you looked at the amounts of enzymes present at different stages in the culture, or have you always harvested cells when a culture has just about come to completion?

Dr. Ruddle: We have done many studies along those lines with another cell population called PK, derived from pig kidney. We find in this established cell line that, if we sample the cells during the lag, log, or stationary stages, there are dramatic changes in the levels of the enzymes that we have studied.

These enzymes in this instance are esterases, LDH, G6PD, and to a lesser extent some of the other dehydrogenases. What we find is—and the results are quite dramatic in the case of LDH—during the lag period for this particular cell line, under the conditions of growth in our laboratory, that the specific activity in LDH may drop as much as eightfold to tenfold during the first 12 hours after subculture. The enzyme activity remains low during log growth and then increases abruptly when the cells become confluent, going back to the original activity levels. So it is important to sample populations at a particular time during the culture cycle to obtain maximum activity. In fact, in the experiments that I have described, the enzymes were extracated from cultures during their confluent plateau stage of the cultural cycle.

I might add that the question of differentiation and modulation which Dr. Harris has discussed is an important consideration for isozyme variants. I restricted my earlier remarks to isozymes of what we might call essential dehydrogenases, without which any cell would probably fail to exist. However, we have studied a number of esterase variants, isozymic variants controlled at three separate loci. We find that these esterases which are common to the kidney and the liver are not expressed in cultured fibroblasts. So in this case the genetic information, although it may be present in the cells, is not expressed. At present one has to restrict oneself to the broadly expressed enzymes which would be expected to be expressed in any cell type.

Dr. C. J. Dawe: I would just like to ask if, in regard to the isozymes you're talking about, you have also conducted studies with some neoplastic mouse cell lines to see if they are equally stable.

Dr. Ruddle: We have looked at neoplastic populations to some extent, and we have particularly looked at plasmacytomas from the mouse which we are interested in adapting to culture. We find that in those cases (these were derived from C3H and also BALB) that they express the isozyme pattern of the strains or origin. We also have examined an erythrocytic tumor, DICR, which was initiated by Dr. Soule; this tumor also has the isozyme pattern associated with the inbred strain origins of these cells.

Characteristics of Primary Cultures and Diploid Cells—Technology of Production

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THE subject of my discussion is the production, handling, and media requirements for primary and diploid cells in tissue culture. I have interpreted my subject not to cover organ culture or cell line tissue culture methodology and will assume that other speakers will cover these subjects during the symposium. In addition, I will limit my discussion to published literature on the subject as well as a limited report on certain technology recently developed in our laboratories. Very thorough reviews of tissue culture methods can be found in excellent publications on the subject (*1–5*). Dr. Joel Warren will discuss the technology of production of primary cells, and Dr. Robert Brackett will cover these aspects of diploid cell propagation.

SOURCE OF TISSUE FOR CELL CULTIVATION

Primary Cells

Kidney tissue from monkey, dog, calf, duck, and whole chick embryo presently provides the major source of primary cells for virus propagation. Because of the importance of obtaining cells free of extraneous, viable microorganisms, many laboratories utilize exhaustive procedures to decrease the possibility of using tissue containing contaminating organisms. For example, chick embryonic tissue to be utilized for production of measles vaccine should come from flocks pretested to insure their freedom from *Mycoplasma*, *Salmonella pullorum*, some leukosis viruses, fowl pox, and avian tuberculosis. These tests need not be done on the flock but can be done on the cultured cells. However, economic considerations usually make testing of the flock mandatory.

Other animals used for sources of tissues, such as dogs and monkeys, are usually held in quarantine for several months before use. During this time, the animal must remain in overt good health and is submitted to a battery of tests designed to minimize the possibility of unwanted agents in the cultured cells from these animals (*6*). Serological tests for certain simian agents are sometimes used to test for the presence of endogenous agents such as SV5 and SV40 (*7*). These workers report a good correlation between positive serology for SV5 and SV40 and the presence of these agents in subsequently cultured cells from the test animals. Bacteriological and parasitological tests of tissues are generally carried out on the tissues themselves to assure the freedom from bacterial and parasitic contaminants (*8–11*).

The age of the donor supplying tissue may be a factor in obtaining a constancy of successful cell cultivations. For example, Marcus (*12*) reported that young dogs provided a higher percentage of positive cultures than older animals. This factor is probably not important to most workers in the field since embryonic or young animals usually provide the source of cells.

Diploid Cells

Man and a variety of animals have been reported as good sources of diploid cells. Puck *et al.* (*13*, *14*) and Swim and Parker (*15*) were the first to report that human and animal cells could be maintained in the laboratory without alteration of morphological or cultural characteristics. Fresh tissue has provided the best source of cells, but good results have been obtained from tissue removed from the whole animal and held for hours at low temperatures (*16*). Adult (*13*, *14*) and embryonic tissue (*16*), as well as malignant tissues (*17*), have yielded diploid cells. Human diploid cell strains (HDCS) have been derived from lung, skin, kidney, heart (*16*, *18*), and thyroid tissue (*18*). Human amnion has not been a good source of HDCS (*18*). Zitcer and Dunnebacke (*19*) indicated that chromosomal changes appeared fairly frequently in cells cultivated continuously from human amnion. Kalk and Hetrich (*20*) have isolated diploid cell lines from bovine lymph nodes with normal characteristics.

METHODS OF CELL CULTURING

Dispersion of Primary Cells

Although the specific methods used in cell culturing vary considerably from laboratory to laboratory, certain fundamental principles are followed throughout for optimal results. They include maintenance of aseptic techniques and rigid controls to prevent contamination from the environment. Following aseptic removal of the tissue or organ from the animal, the tissue is carefully cleansed of blood, fat, and other unwanted tissue. The tissue is then minced and washed again before dispersing the cells with trypsin (*21*). A variety of containers have been used for holding cells during dispersion including a Waring blender (*22*) or an indented Erlenmeyer flask

(*23*). There are, no doubt, numerous other methods, but in our laboratories, we have found the fluted Erlenmeyer flask to function very efficiently. In general, with bovine tissue, we have used a relationship of tissue to container size of 1 calf kidney to a 500 ml flask to 3–6 calf kidneys per 2-liter flask.

When the overnight trypsinization procedure is used (*24*), the fragmented tissue is briefly washed in cold, buffered trypsin in saline 3 times to remove fat and waste before incubation at 4°C overnight in this solution. The next morning, the dispersed cells are filtered through cheesecloth (to remove clumps and connective tissue) into growth media (GM) containing serum, washed twice by centrifugation at low speed, and diluted on the basis of packed cell volume (PCV) in growth media for planting. For immediate processing, the tissue is handled as described above except that repeated extractions are done at 37°C. Cells are constantly removed to GM to neutralize trypsin until the tissue is exhausted.

We have found that we can hold minced bovine kidney tissue for up to 4 days before dispersion when the tissue fragments are kept at 28°C under constant agitation. After these cells are held for lengthy periods, they can readily be extracted with trypsin at 35°C. Yields of BK cells range from 25–35 ml PCV/kidney or 50 ml of PCV/100 g of cortical tissue (*25*). Approximately 1–1.5 ml of cells are usually obtained from monkey kidney by the overnight or continuous extraction procedures.

Methods for preparing chick and duck embryo cells for cultivation are generally similar, except that cell dispersion occurs rapidly, and, therefore, a shorter period of trypsin extraction is required. Direct cell counts are usually made on extracted DE and CE cells and they are planted at 300,000 and 200,000 cells/ml, respectively.

Hayashi and LoGrippo (*26*) reported that pancreatin has distinct advantages over trypsin for dispersion of human amnion due to the mucolytic activity of this enzyme. Since all dispersing agents will act on cells as well as their supporting structures, the cells would be destroyed if left for a long period in contact with the enzyme. Therefore, all dispersing procedures are established to bring about cell release with a minimal destruction of cells.

Some attempts have been made at automation and simplification of the various procedures of cell dispersion (*27*, *28*). However, a limited survey of my fellow workers in the industry indicates that manual procedures remain in general use and are the most efficient procedures at this time.

Cell Planting—Primary Cultures

After cells are extracted from the specific organ or whole embryo, they are diluted to a special concentration in GM. The number of whole cells may be determined by direct counting in a hemocytometer or electronic counter, or estimated on the basis of nuclei count. In addition, the cell count may be estimated on the basis of PCV. In the latter procedure, the dispersed cells are centrifuged at a constant gravitational force in a graduated container. A ratio of cell count to PCV can be developed or planting efficiency determined on the basis of experimental determinations at various dilutions of PCV. Chemical methods probably offer the greatest accuracy of all the above-mentioned methods. Primary cells are usually diluted to a PCV of 1/200 to 1/600 for planting. In general, these dilutions represent a final concentration of approximately 200,000–600,000 cells per ml.

The containers generally used for planting primary cells are made of both glass and plastic. Disposable glass tubes and bottles have been particularly useful, as their use eliminates or minimizes the costly and difficult problems of glassware washing. However, if pyrex or other more permanent glassware is used, it must be thoroughly washed and carefully rinsed before use.

For many years, the pet scapegoat for the failure to grow good cells has been improperly cleaned glassware or impure water. In our laboratories, when growing primary cells, we routinely use a disposable glass that normally is not washed before use. When growing HDCS, however, we first wash the glassware in mild detergent, then thoroughly rinse it in tap water, and rinse it again in distilled water.

Various sizes and shapes of glass and plastic containers are used throughout tissue culture laboratories, depending on the specific needs. After the cells are planted, the containers may be incubated in a stationary position, or rocked or rolled. In closed systems a CO_2-buffered medium is normally used, whereas in open systems an incubator flooded with 5% CO_2 in air is used. We have recently introduced an apparatus for mass cultivation of monolayer cultures. With this equipment a multiple-tier structure is used in a closed system.

The evolution of this equipment originally was based on the simple observation that the amount of virus produced in cell culture was a function of the amount of cells in the container. The basic structure of this multisurface mass-scale tissue culture propagator is shown in figure 1. Units ranging in size up to 200 liters consist of an enclosed vessel containing a multiplicity of separated glass plates or discs upon which the cells attach and proliferate. Means for mixing and aeration of the media are provided. Sample ports facilitate the addition of cultures and media, withdrawal of samples, washing of cell monolayers, and harvesting of cells and cell products. Various types of cells have been grown in the mass propagator, including calf kidney, HDCS–WI–38, neonatal human foreskin, BHK21, and strain L. The primary advantages of this system over the bottle method to date are decreased bacterial and PPLO (pleuropneumonia-like organisms) contamination rates, less chance for cross-contamination of cells, simplicity of handling, and resultant cost advantages (*29*, *30*).

Cell Planting—Diploid Cells

Fresh tissue has provided the best source of HDCS, although Hayflick (*16*) has been able to grow active cells from tissue fragments held for as long as 5 days at 4°C. Both fragments and trypsin-dispersed cells have been used successfully for originating diploid cell growth. The size of the container used for planting the original tissue is generally determined by the amount of tissue available. For example, Hayflick (*16*) reports that he has usually dispersed lung fragments from one 3-month-old human fetus into 4 Blake bottles. Growth medium is added, and incubation is at 37°C until visible growth is evident. Cells are then fed and reincubated until a confluent sheet is formed. This usually requires about 10 days. The cells are removed from the glass surface with trypsin, aspirated, and split 2:1 in fresh GM and subcultured in new bottles. Four to one splits are also used in some laboratories.

The multiplying cells show contact inhibition, and thus a monolayer only will be obtained under routine conditions. Kruse and Miedema (*31*), however, report that the contact inhibition can be overcome by regular perfusion of growing cells. They indicate that as many as 17 layers of cells have been produced in their special perfusion apparatus.

Perhaps the most critical stage in the handling of HDCS relates to the need for subculturing on a regular basis after each 3–4 days of incubation (*16*, *32*). Under such conditions, the HDCS are considered useful for virus vaccine production through the first 30 passages (*33*). The normal lifespan of HDCS is generally reported (*16*) to be approximately 50 passages, although other investigators report a much more limited lifespan (*18*). Prior to the time the cells begin to lose their capacity to multiply, they will generally sheet out within 3–4 days following a 2:1 split. In subsequent passages the cells multiply more slowly and therefore are not ready for splitting until sometime later.

The normalcy of the chromosome pattern of HDCS has been demonstrated repeatedly (*13–18*). Examples of chromosome analysis for HDCS–WI–38 cells grown in stationary bottles, rolled bottles, and the mass propagator are shown in table 1. These results that are demonstrative of the left skewness from 2N of 46 are as expected, due to the technical problems of preparing cells for counting. The failure to find any consistency of missing chromosomes also is generally interpreted as representing a normal finding.

Although Hayflick (*16*) reports that HDCS is generally free of PPLO, we have found many lots of WI–38 cells contaminated with such organisms. Occasionally, we have been able to eliminate PPLO by repeated treatment of cultures with combinations of antibiotics. However, no one specific treatment appears to always be effective in curing infected cells. Once free of PPLO, constant care must be taken to keep the organisms from recontaminating the cultures. We have found that complete elimination of mouth pipetting, covering of the face and head, and the use of sterile gowns to minimize the chances of such contamination have aided in decreasing the incidence of reinfection of WI–38 cells with PPLO.

TABLE 1.—Karyologic analysis of WI–38 cells grown in different containers

Cell lot	Cell passage used	Equipment for cell propagation	Karyotype analysis	Comments on analysis
A	24–25	Stationary bottles....	Normal......	
B	23–26	Stationary bottles....	Abnormal....	High frequency of breaks and two abnormal karyograms.
C	23–25	Stationary bottles....	Normal......	
D	25–26	Stationary bottles....	Normal......	
E	28	Stationary bottles....	Abnormal....	Marked aneuploidy—Mold contamination—would not have been acceptable with 1966 recommendations.
F	27	Mass propagator.....	Normal......	
G	23	Stationary bottles....	Normal......	
H	32	Stationary bottles....	Normal......	Directly from freezer.

Cell stocks of HDCS may be frozen at any time during the rapid growth phase. Addition of 1.5–2.0 × 10^6 cells to 10% glycerine in GM, followed by slow freezing, has generally been a satisfactory procedure (*16*). The cells are rapidly thawed when they are to be used. The cells retain their normalcy after freezing in terms of chromosome analysis, morphology, virus spectrum, etc. (table 1).

Media

Media used for propagation of tissue cells are usually divided into growth media (GM) and maintenance media (MM). Eagle (*34*) reported that the growth requirements for HDCS and primary cells were basically the same. The GM for propagation of primary cells usually contains an animal serum. Calf or fetal calf sera are used extensively in concentrations varying from 2–20% depending on the specific cell system employed. Horse, monkey, and rabbit sera have also been used extensively, although these sera have been reported to be toxic to HDCS (*17*, *35*). Most early investigators used serum fortified by addition of various tissue extracts (*36*, *37*). These extracts have gradually been replaced in most laboratories by synthetic media such as 199 of Morgan (*38*) or Eagle's (*39*) in a balanced salt solution (Hanks' or Earle's) (*40*, *41*) and with sodium bicarbonate to provide a CO_2 buffer system. Healy and Parker (*42*) have recently reported on an improved synthetic medium called CMRL-1415 which they find useful for cultivation of newly explanted mouse embryo cells. This medium may deserve consideration for tests with HDCS. Natural products such as lactalbumin hydrolysate (LH) also are used extensively with sera as GM (*43*).

Eagle's basal medium (BME) (*39*) with 10% unheated calf serum has been used almost exclusively for growth of WI–38 cells (*16, 44*). Hozinski *et al.* (*45*) have isolated an HDCS from human lung with a medium containing Eagle's solution with 0.5% LH. Jacobs (*46*) found that only the amino acids L-cysteine and L-glutamine were essential to growth of WI–38 cells, exclusive of other amino acids. In addition, galactose could be substituted for glucose to produce a more stable *p*H control in the absence of a CO_2 buffer system. Ferguson and Wansbrough (*17*) have used medium 199 with 5% fetal calf serum with good results. Rappaport *et al.* (*35*) report good success in culturing diploid cells with a medium composed of Eagle 1X, LH 30%, 10% cow serum in Hanks' salt solution.

Dried, blended media were found by Swin and Parker (*47*), Greene *et al.* (*48*), and Hayflick (*49*) to have advantages of reproductibility, stability, and reduced cost over preparations of liquid media, since larger batches of the dried media can be prepared and held for long periods before use.

Following the growth of cells to a confluent sheet, the GM is removed, and the cell sheet is washed with buffered saline and replaced with maintenance medium (MM). The most frequently used MM's are 199 or BME, or combinations of the above. Antibiotics generally are added to both GM and MM just before use. Although antibiotics have been used to rid cultures of bacteria (*50*), such as the use of penicillin to remove leptospira from canine kidney, this does not appear to me to be an acceptable method. Certainly, initial freedom from such contaminants is a much safer procedure.

Medium sterilization is generally accomplished by autoclaving stable constituents and filtration of unstable substances. Filtration through asbestos filters should be avoided due to release of toxic components from the filter (*51*). Cellulose filters have generally been acceptable. Ethylene oxide also has been reported useful for sterilization of media (*52*).

SUMMARY

The technology of producing primary tissue cultures has been developed to a degree that permits the production of cells at a consistent level. Since many organs, particularly from adult animals, contain microorganisms, the major problem of supplying useful cells is not the manipulation of the tissue or nutritive requirements of the cells, but the procurement of a clean tissue with which to work. The most effective approach to solving this problem appears to be the use of isolation and quarantine of the herd coupled with serological and microbiological tests and eradication of infected animals from the herd.

Important reasons for the value of human diploid cells for virus propagation are these: The cells can be frozen and held until ready for further use; the karyotypes are stable; the cells are capable of multiplying in culture for many passages; the cultures are free from extraneous agents; there is a broad virus spectrum.

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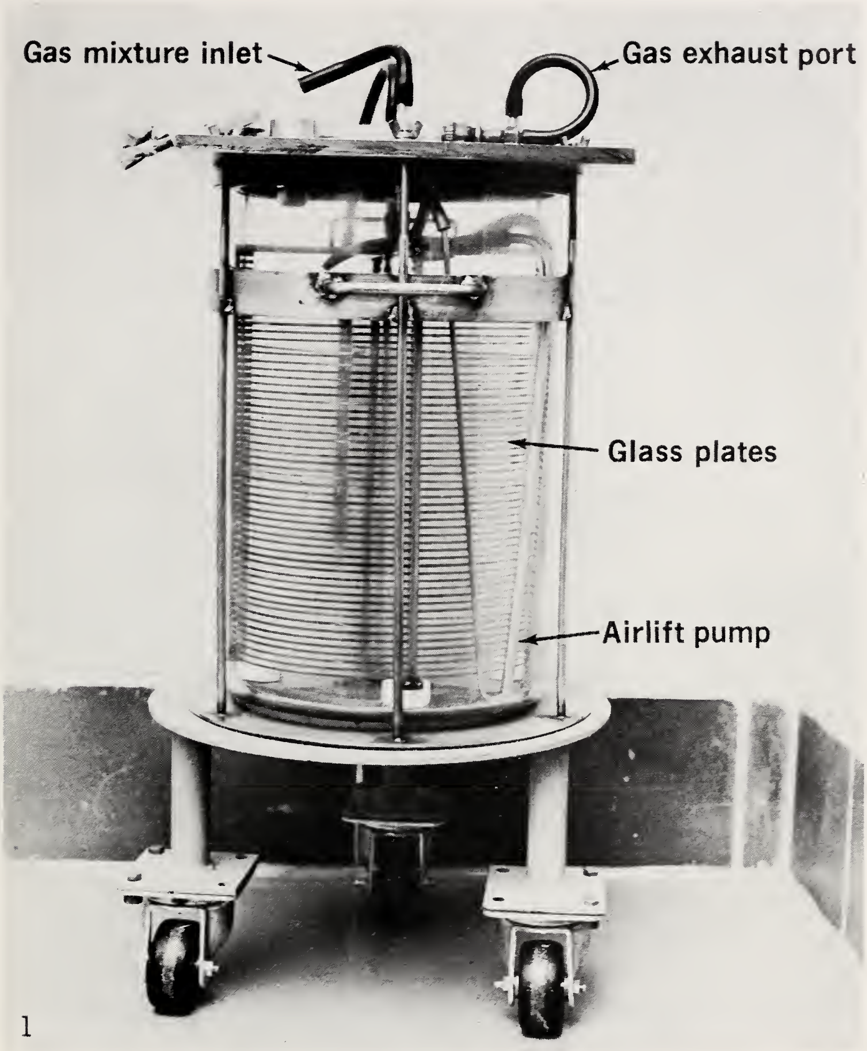
Gas mixture inlet
Gas exhaust port
Glass plates
Airlift pump
1

FIGURE 1.—Multisurface tissue culture propagator.

Technology of Production of Primary and Diploid Cell Cultures

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MY comments will be quite general, and I will "key-in" on only three points from Dr. Holper's paper.

Those of us involved in the manufacture of vaccines for administration to humans expend a tremendous effort to assure the safety of our products. Safety is a multifaceted problem. However, one item of prime concern is the possible presence of adventitious agents in the cell culture system used for virus propagation. We will hear a great deal more about this in later sessions of this Conference.

I want to emphasize one aspect—that of the number of individual animals which serve as tissue donors. With a few possible exceptions, the tissue of more than one animal contributes to the manufacture of each vaccine lot. Most monovalent and polyvalent virus vaccines are produced on the tissue from two to a thousand or more donors. The likelihood of an adventitious virus being introduced is proportional to the number of animals used. The selection of donors from carefully controlled flocks or colonies substantially reduces this factor. Using a diploid cell line derived from a single individual for the preparation of numerous lots of vaccine should reduce even further the risk of viral contamination.

This brings me to my second point, the matter of the quantity of cells available from a diploid cell line. Hayflick (*1*) has pointed out that the potential of WI–38 for production was "billions of doses of dozens of kinds of human virus vaccines." My experience with WI–38 is relatively small. However, extensive work with diploid cell lines derived from human embryonic lung developed in our laboratories verifies that the potential for this type of culture is indeed very large. Our diploid cell lines have not regularly exceeded forty-five population doublings, but this is not a matter of concern since vaccine manufacture is quite practical at passage levels between 25 and 30.

The use of improved nutritional conditions through which greater growth of WI–38 has been achieved, such as those described by Baugh and Tytell (*2*), offers a promise of even greater cell yields. However, these conditions need to be further explored before their utility for production can be fully assessed.

Finally, the fact seems clearly established that human diploid cell lines, when frozen slowly, stored at liquid nitrogen temperatures, and thawed rapidly, still retain the properties they possessed before they were frozen. This technique affords the opportunity for extensive study of the cells before their ultimate utilization. With advances in cryobiology and improved cell culture methods, this may be achieved with kidney cells from many mammalian species. A trypsinized cell suspension is prepared and a sample is removed for testing. The bulk of the cells are then frozen at a controlled rate, stored in liquid nitrogen, and may be rapidly thawed when tests have shown them to be free from contamination. In our laboratory high rates of recovery routinely have been obtained. In this manner pretested cells can be used to produce primary cultures with no apparent difference from unfrozen cultures.

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Industrial Production of Primary Tissue Culture

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AS pointed out many years ago by Philip White, all of the basic ingredients for preparing modern tissue cultures were given by William Shakespeare in *Macbeth*, Act IV, Scene 1.

"Round about the cauldron go:
In the poison'd entrails throw.
Toad, that under cold stone
Days and nights has thirty one
Swelter'd venom sleeping got,
Boil thou first i' the charmed pot."

"Fillet of a fenny snake,
In the cauldron boil and bake;
Eye of newt and toe of frog,
Wool of bat and tongue of dog,
Adder's fork and blind-worm's sting,
Lizard's leg and howlet's wing."

"Scale of dragon, tooth of wolf,
Witches' mummy, maw and gulf
Of the ravin'd salt-sea shark,
Root of hemlock digg'd i' the dark,
Liver of blaspheming Jew,
Gall of goat, and slips of yew
Sliver'd in the moon's eclipse,
Nose of Turk and Tartar's lips,
Finger of birth-strangled babe
Ditch-deliver'd by a drab,
Make the gruel thick and slab:
Add thereto a tiger's chaudron,
For the ingredients of our cauldron."

"Cool it with a baboon's blood,
Then the charm is firm and good."

A minimal estimate of the total volume of primary monkey kidney tissue culture commercially produced in the United States since 1954 is:

Monolayers	950, 000 liters
Maitland-type cultures	750, 000 liters
Total	1, 700, 000 liters

As table 1 shows, this has been primarily for poliomyelitis vaccine, with lesser amounts for measles and adenovirus vaccines. The basic purpose of the production of such cell cultures is to make viral nucleoprotein in the highest yield and in the shortest possible time. Hence, most of the current technology has been developed empirically and tailored to the requirements for a particular end product. As Mazia has aptly stated: "Metabolic studies of dividing cells at a level worthy of modern biochemistry have scarcely begun."

TABLE 1.—Current status of biologicals prepared in tissue culture

Tissue culture	Live	Killed	Experimental	Licensed or adopted
Suspension (Maitland)				
Poliomyelitis		+	....	+
FMD		+	....	+
Monolayer				
Poliomyelitis	+	+	....	+
Measles	+	+	....	+
Adenovirus		+	....	(+)*
Mumps	+	....	+	....
Rubella	+	....	+	....
Vaccinia	+	....	+	....
Adenovirus	+	....	+	....
Yellow Fever	+	....	+	....
Arbor B	+	+	+	(+)†
Miscellaneous respiratory		+	+	....
Veterinary: ICH, IBR, BVD, Hog Chol.,‡ etc	+	+	....	+

*Use has been suspended.

†Foreign manufacturer only (RSSE).

‡ICH=infectious canine hepatitis; IBR=infectious bovine rhinitis; BVD=bovine virus diarrhea; Hog Chol.=hog cholera.

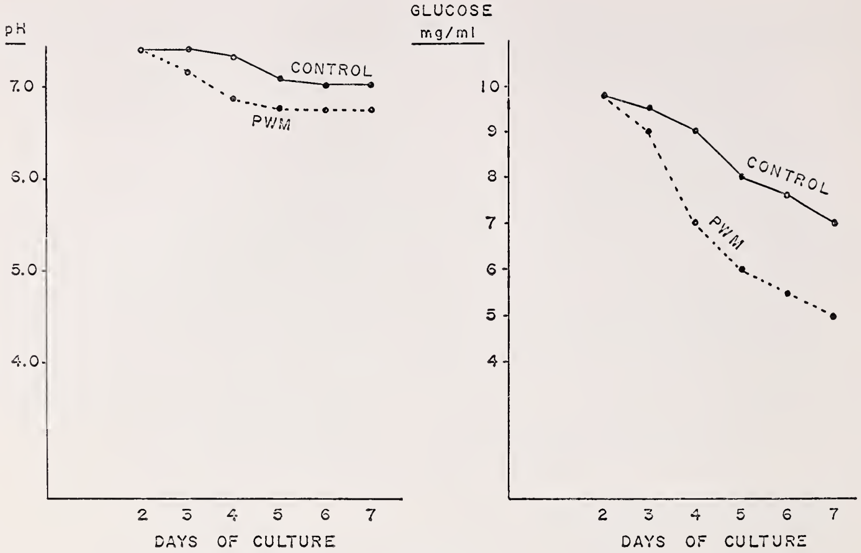
In the industrial production of primary cells, we have to balance the two objectives of maximal plating efficiency with the optimal yield of a reagent. Table 2 summarizes the major factors which modify these. Dr. Holper has discussed most of these factors. We would stress the particular importance of age and health of the donor animal and the use of complete asepsis in the removal of tissues. The choice of medium for a set of culture conditions is determined by the need to reduce manipulation of large containers, as in the changing of media and adjustment of pH. Similarly, the type and concentration of added serum are often dictated by economic factors. It is the general experience of most laboratories that exposure to trypsin or other dispersing agents should be for as short a time as possible.

TABLE 2.—Critical factors in cultivation of primary tissues for vaccines

Plating efficiency factors	Viral yield factors
1. Tissue donor: Species, genetic strain Age and nutritional state Organ Acclimation and quarantine	1. Infective agent: Strain Adaptation to cell substrate
2. Medium: High protein Adequate buffering Supplementary growth factors Ratio to volume of cells	2. Growth characteristics: Multiplicity of input Rate of multiplication Rate of cell destruction, if any Single or multiple harvests Relation of immunogen to infective virion
3. Container: Stationary vs. moving Pre-wash and sterilization procedures Ratio of gas to fluid volume	3. Miscellaneous: Latent interfering (or helper) agents Media volumes Temperature and *p*H
4. Techniques: Fresh or frozen cells Cell dispersion methods Temperature and *p*H Cell seeding concentration Media changes	

Three recent developments deserve brief mention because of their importance to large-scale preparation of primary cultures. These are the use of increased amounts of adenosine triphosphate in the growth medium (Steiner), the addition of aluminum chloride to control adventitious virus proliferation (as suggested by Wallis and Melnick), and, finally, the administration of compounds, capable of accelerating growth of tissues, to donor animals before they are killed. One example of this is the use of pokeweed mitogen which we have found dramatically accelerates growth of lung and kidney tissue culture when it is parenterally inoculated into a donor 3–5 days before removal of tissues. Examples of the accelerating effect of pokeweed mitogen are shown in text-figure 1 and figure 1A (*see* fig. 1B for comparison).

In conclusion, there are already indications that the next generation of tissue culture vaccines may be prepared from purified, and not crude, virus, and that only certain nucleoprotein or protein fractions may be utilized. To keep the cost and availability of such highly refined biologicals within reason, we may be forced to abandon our present, highly laborious manual procedures of tissue culture production and adopt engineered and automated methods of greater predictability.



Text-figure 1.—Hydrogen ion concentration and glucose consumption curves as indexes of the growth of lung tissue culture from newborn guinea pigs. One group of animals was inoculated with 10 mg of pokeweed mitogen.

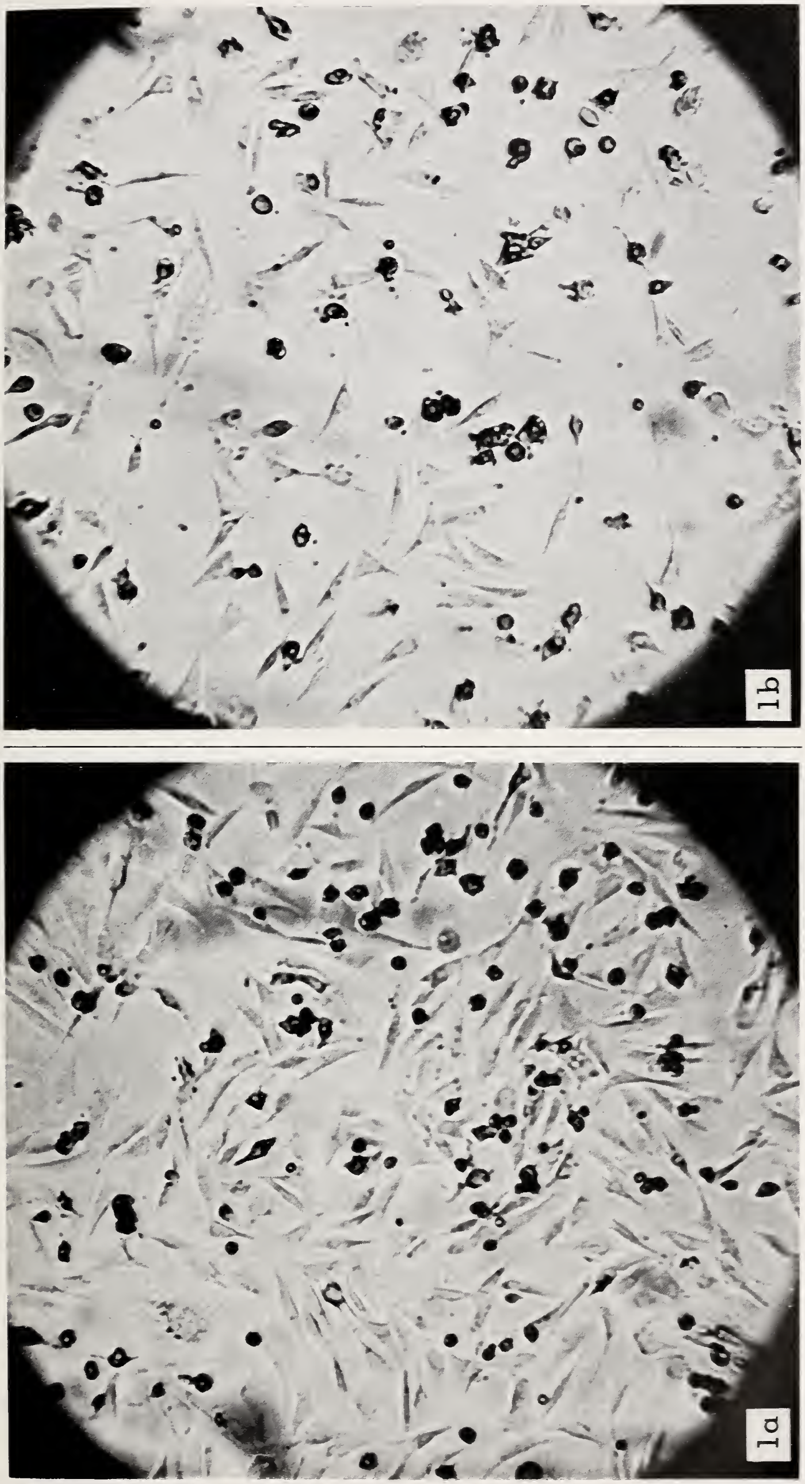


Figure 1.—Guinea-pig lung culture, 5 days. Growth of guinea-pig lung cultures 5 days after explant from an animal treated with pokeweed mitogen (fig. 1A) compared to that from a control animal (fig. 1B).

GENERAL DISCUSSION

Dr. S. J. Pirt: What exactly is the pokeweed mitogen?

Dr. J. Warren: Pokeweed mitogen is an extract of the plant *Phytolacca americana.* It was found by Barker and Farnes to stimulate the mitosis of leukocytes *in vitro* in the same manner as phytohemagglutinin. We abbreviate it "PWM." It is commercially available.

Dr. J. H. Hanks: The idea of the PWM is really intriguing when you think about the many problems in infectious disease. Has anyone been bold enough to try the PWM at the onset and during the course of different microbial diseases?

Dr. Warren: We have only preliminary results, but the rate of rubella virus multiplication in cultures derived from pokeweed-treated donors is dramatically increased over that in controls from untreated animals. I realize that this does not answer your question—that is, whether you would modify resistance or susceptibility with pokeweed—but it is the only information we have on this point.

Dr. Wheelock and Dr. Friedman have been studying these in relation to interferon production.

Dr. H. Toolan: Have you used phytohemagglutinin as well as PWM for this purpose, and was your dosage given parenterally?

Dr. Warren: Yes, we have used both and find that PWM is more effective. The dose we have used, based largely on our experience with guinea pigs, is 1/10,000th the body weight.

Dr. P. Gerber: How does the mitogen affect the karyotype?

Dr. Warren: We don't know.

Dr. K. K. Sanford: I have two questions. First, does the mitogen increase the mitotic rate of cells other than the leukocytes or lymphocytes in culture?

Second, how does the yield of cells in the multilayered tissue culture propagator compare with that in the packed column-type cultures?

Dr. Warren: If I understand the question, do cultures derived from pokeweed-treated animals show increased mitotic activity? Is that correct?

Dr. Sanford: No, I understand they do, because you said that you get better growth in them. But I wondered if the increased mitosis was limited to the lymphocytes.

Dr. Warren: To our surprise we do not see increased mitotic activity in cultures made from pokeweed-treated donors.

Dr. Sanford: You are just getting better survival?

Dr. Warren: We are finding a more rapid rate of multiplication. We are getting an accelerated metabolic rate. But we do not see any abnormal increase in mitotic activity in cultures of lung, kidney, and liver. Now, we have not done anything with leukocyte culture. These have been primarily lung, kidney, liver, and so on.

Dr. Sanford: How do you get increased cell numbers without increased mitotic rate unless you are having better survival?

Dr. Warren: Perhaps I didn't explain myself properly. We are not seeing an abnormal appearance of deranged mitotic figures. We are getting essentially normal mitotic patterns in rapidly developing cells.

Dr. Sanford: I wondered, of course, because the lymphocytes can contribute quite substantial numbers of cells to a primary culture; if these are undergoing maybe one division, that would make it appear that you were having quite marked growth, and I wondered if you were getting increased growth in the fibroblasts and some of the other cells too.

Dr. Warren: No, this increased growth involves primarily pulmonary epithelium cells, as shown in figure 1 of my paper.

Dr. J. C. Holper: The cell propagation in the multiscale propagator has given roughly comparable results to the stationary bottle culture in terms of growth of cells as well as viral multiplication.

Dr. C. J. Dawe: Dr. Holper, what level of aneuploidy do you accept as being within the definition of normal karyotype in your cultures? In other words, what proportion of aneuploid cells may you have, and still accept it as a diploid cell line?

Dr. Holper: We have followed the recommendations from the meetings in 1966 of the Wistar Institute of Anatomy and Biology and allow 20% aneuploidy.

Dr. I. S. Johnson (Lilly Research Laboratories) : Dr. Warren, is there a change in the length of generation time of the mitogen-treated cells—from mitogen-treated animals—or is it the same? I think I am having some of the same difficulty Dr. Sanford did.

Dr. Warren: We would assume so.

Dr. Johnson: From the mitogen-treated animals compared to the other?

Dr. Warren: I can't answer that.

Dr. Johnson: I was wondering if the generation time in the cells from untreated animals was 16 hours and from treated was 12 or—

Dr. Warren: Up until now we have analyzed this by relatively crude indexes of the time for complete dispersion, glucose consumption, *p*H change, and so on. We don't know anything at all of what you are asking—that is, cell generation time for individual cells. We don't know anything about diploidy, and so on, and particularly the intriguing question of oncogenic potential resulting from this kind of treatment.

Dr. A. J. Kniazeff: We started using pokeweed in cultures of lymphocytes and fibroblastic cells. We do get an increased mitotic rate but not an increased growth rate. The changes we have observed—this is with canine tissue—are both in the leukocyte and the fibroblast.

Dr. Warren: I didn't realize that this particular issue would be so interesting. But I want to stress something: The addition of pokeweed extract to monolayer or suspension cell cultures of lung or kidney has no effect whatever. Only when we give the material to the donor animal—and we must give it no less than about 3 days before the animal is killed—do we see this stimulating activity. It is not the same situation as with a culture of leukocytes, where one can add mitogen to the culture system and obtain a blastic effect.

Dr. R. Haff (Smith, Kline and French) : Dr. Holper, how did you get cell distribution on implanting in your multisurface structure?

Dr. Holper: These are good cell distributions. We use a stirring apparatus to disperse the cells as they are added to the propagator in the beginning; this allows the cells to distribute themselves throughout the surface area, and then this is halted and, of course, can be reinstituted at any time.

We have used a variety of flow systems in the total system. We can remove the total amount of fluid, for example, through a second system and replace the fluid with fresh fluid, and that sort of thing.

Dr. P. D. Cooper: Dr. Holper, do you have to strip your cultivator down each time or can you harvest and then reseed without cleaning it up in between?

Dr. Holper: For most of the work that we are doing we are interested in harvesting all of the fluids at one specific time, although you can, of course, remove all the fluids to a separate container and replace them with fresh fluids if you would like at any specific time. And, of course, we have sampling ports so you can take samples at intervals. Does that answer your question, sir?

Dr. Cooper: Not quite. Could you give us a brief rundown on how this thing is operated for primary cultures?

Dr. Holper: The propagator is used as we use cell bottles, in the sense that we add a cell suspension, diluted essentially the same way we do for stationary bottles, to the container. The cells are stirred with a stirring bar at the bottom of the propagator during this interval until they are dispersed throughout the container.

Then they may be incubated in a stationary position without stirring, or they can be stirred, depending on your specific needs.

Cells disperse—or the flakes disperse—in about the same length of time as it takes for a stationary bottle, and then they are ready for virus inoculation. The fluid can then be removed, replaced with virus inoculum—as is done in primary cultures—and then harvested at various intervals. Samples can be taken, of course, and titered as you go along.

For diploid cells, the cells, of course, are removed with trypsin, as you do stationary bottles.

Dr. Cooper: Are they cleaned before reseeding?

Dr. Holper: Yes. Then we take apart and clean the whole container.

Identification of Established Cell Lines With Reference to Karyology[1]

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Every researchist using cell cultures knows that the HeLa cell line, which he may obtain from the laboratory across the hall as a replacement for his own contaminated stock, may fail to exhibit some property he regards as characteristic of "*The HeLa line.*" If one ignores for the moment the problem of accidental contamination with other mammalian cell lines, cell populations of common descent which have been propagated separately tend to drift apart in terms of their culture characteristics and their genetic content. Such a divergence occurs even within the *in vivo* somatic cell population in terms of differentiation. This divergence is a necessary consequence of any branching process of growth with uniparental generation, a concept which is central to the accepted definition of the "cell line":

> "The term 'cell line' implies that cultures from it consist of numerous lineages of the cells originally present in the primary culture." (Proposed Usage of Animal Tissue Culture Terms. *J Nat Cancer Inst* 38: 607–611, 1967.)

Similarly, within the definition for cell line as given by Ross, Treadwell, and Syverton (*1*):

> In theory the "line" may be conceived as a succession of cellular descent consisting of one parent per generation. By this concept, any cell population represents a collection of clonal lines diverging increasingly with time in degree of relatedness with respect to the donor inoculum. Depending on its origin, any variation inherent to the donor inoculum will be segregated increasingly in the progeny lines.

Since the ability to resolve differences between cell lines depends on the existence of distinctions which are relatively invariant, we must determine the nature of this inherent variation in the origin of a line and how it is "segregated increasingly."

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As emphasized in the terminology adopted for this Conference, the cloning of a cell line does not necessarily confer homogeneity on the resulting population. However, it is obvious that if the original cell replicates its genome with absolute faithfulness and distributes the products equally to its daughter cells without mishap, the population would be homogeneous, at least genetically. Therefore, variations which are to be "segregated increasingly" during propagation include not only the input variation, such as gene heterozygosity between homologous chromosomes, but also variation acquired during continued expansion.

A constitutive change, as by gene mutation or by the deletion of a segment of a chromosome, would represent a departure from the parental type which would be segregated as a distinct sector, as one clonal line in a "collection of clonal lines." A distributive error, an unequal chromosomal contribution to the daughter cells in a single anaphase, would also initiate a new sector. Actually it would mark the origin of a "twin spot," 2 new clones within the mass population. An appreciable frequency of chromosome breakage or a tendency for error in anaphase distribution constitute the general problem of instability, which is the foremost factor affecting identification or maintenance of integrity of a cell line for whatever research purpose proposed. Karyological studies provide the means for assessing new variability being introduced as well as the evidence for departures from parental form which may have occurred in the history of the line.

Distinctions in terms of karyology are limited by problems of artifact, such as cell rupture and loss of chromosomes, by variation in the length of the chromosome arms due to differential compaction, and by the tediousness of the microscopic work itself. In spite of this, certain useful determinations can be made through even a cursory examination of the metaphase chromosomes of a cell line. In early papers published on the identification of cell lines with anomalous properties suspected of being contaminants, crude distinctions between general morphological classes of chromosomes could in every case confirm the interpretation as to species of origin made from serological and transplantation evidence (*2–5*).

The usual problem the karyologist has is to determine the species of origin of a given cell culture. If the most probable sources of cell contamination or confusion of labels are known, a karyological answer can be critical. For example, a line offered to our laboratory for confirmation of its supposed human origin could readily be determined as a mixture of mouse and chicken cells by the examination of only 20 metaphase cells. Both of these cell lines were mixoploid, but the identification as to species was highly probable, since bird cells possess microchromosomes and the chromosomes of the mouse are entirely telocentric. Statistically, of course, this could not exclude the possibility that human cells maintained a foothold as a minor element in the population; nor could it exclude the possibility that these lines originated, respectively, in the tissues of a lizard and a cow. Thus, karyological identifications are limited to broad probabilities in the case of mixoploid lines.

Similar considerations apply in the identification of a particular cell line on the basis of a marker. The term "marker chromosome" usually refers to an abnormal chromosome with relatively distinct morphological characteristics. However, there is also the problem with certain markers that cannot be distinguished even though they may have independent origins. An example of this would be the relatively poor marker formed in human cells upon the deletion of the long arm of chromosome #1, beyond the locus of the secondary constriction normal for #1. Again, evidence for identity is based only on the additive probabilities of various features.

For comparisons of classically diploid cell lines, the identification can be quite precise. Human diploid cell cultures could theoretically even be identified individually on the basis of a minor karyological variation, such as a difference in the length of the Y chromosome; however, these variants are rare, are difficult to evaluate, and require the examination of a large number of cells. Distinctions between diploid cell lines of the same species can usually be made readily on the basis of heteromorphic sex chromosomes; this approach has proved quite useful.

In the same way that ordinary diploid cell lines can be identified with more precision, the defined aneuploid lines, such as those that may be derived from the skin biopsy specimen from a case of trisomy, are easily identifiable. In general, researchists working with nonpermanent diploid and with defined aneuploid lines find that such lines are highly stable, at least until the latter part of their limited period of growth *in vitro*. Even in this degenerative period of growth *in vitro*, a predominant part of the population is euploid, or the same as the tissue of origin in the case of the defined aneuploids.

This raises an important point of confusion in discussions of the karyological character of any cell line. Terms, such as diploid, aneuploid, etc., are derived from usage in plant and animal cytogenetics; their application to cell lines is not always appropriate, but we must communicate with each other. For example, to use the term "diploid" to refer to a cell line because the most common class or stem cell karyotype is diploid is improper if a significant portion of the population consists of karyotypes which can only be interpreted as biologically aneuploid or pseudodiploid.

However, until now, nearly all of the established cell lines in research use were highly mixoploid. In the American Type Culture Collection booklet, *Registry of Animal Cell Lines*, 60 mammalian lines are listed, and only the DON and the DEDE Chinese hamster lines are considered diploid, with a small or negligible frequency of abnormalities. However, these lines were characterized at their 28th and 29th passages *in vitro*, and a similar characterization as "classic diploid" might perhaps have been assigned even to HeLa at those passages *in vitro*. In our laboratory the DEDE line showed a variety of novel karyotypes in more than half the cells of the population before the 60th population doubling *in vitro.*

Theoretically we may conjecture that the unstable mixoploid cell line retains a great amount of genetic variability through a reshuffling and remodeling of its chromosomal content, and that this plasticity provides a broad basis for survival in the varying conditions of tissue culture. On the other hand, there is a case of a permanent rat cell line which retained its original diploid configuration even after 300 or so population doublings *in vitro* (*6*). Interestingly, there is a similar high stability of diploid number and karyotype in a second rat cell line reported in the October 1967 issue of *Mammalian Chromosome Newsletter* (vol. 8, No. 4). This would indicate that there is no absolute dependency upon karyological remodeling for long, continued propagation *in vitro*, at least for some cells.

Recently, lymphocytic cells isolated from Burkitt's African lymphomas have been shown to be diploid, near-diploid, and pseudodiploid in their chromosome constitution after cultivation for extended periods *in vitro* (*7–9*). The study of such lines during continued propagation *in vitro* has in some cases revealed a fall from grace, as evidenced by the emergence of a novel rearrangement or "marker." The retention of such a translocation or other rearrangement then yields an apparently quasidiploid line. In general, these lines may be regarded as moderately stable, since there is usually either no departure from diploidy or there is a single alteration in number or form with little further tendency to instability. Other examples of relatively high stability in established cell lines are available from earlier work by Ruddle (*10*), in the quasidiploid RPMI-2650 human cell line (*11, 12*), and in a quasidiploid rabbit cell line described by Puck, Waldren, and Tjio (*13*).

Although many workers have attempted to stabilize a highly mixoploid population by cloning, and thereby to obtain a variant which does not continue to exhibit changes in chromosome form or number, few have been successful (*10, 14*). Thus there may very well be a fundamental difference between the highly stable pseudodiploid or diploid permanent lines cited above and the permanent mixoploid lines.

Apart from the intrinsic factors affecting stability, the variety of extrinsic agents which may be acting on a cultured line *in vitro* to divert it are numerous within three major areas: viruses, chemicals, and irradiation. *In vitro* infection with a virus such as simian virus 40 (SV40) can reproducibly transform diploid, nonpermanent, human cell lines into established, mixoploid, highly unstable lines (*15*). However, in some cell-virus systems, wherein oncogenicity can be assayed in the animal, no direct correlation between the karyological instability and tumor formation is apparent (*16*).

Although viruses occupy much of our attention, it is now clear that even *Mycoplasma* infections (*17, 18*) or physical cooling of a culture to 5°C (*19*) can induce significant amounts of chromosome breakage.

Most interesting in respect to the extrinsic factors which may affect chromosomal stability of cells in culture are the recent findings from the National Cancer Institute concerning serum (*20, 21*). The use of fetal calf serum in place of horse serum appears to delay or to inhibit entirely spontaneous neoplastic transformations *in vitro*, as measured by inoculation of cultured mouse cells into appropriate hosts (*20*). Similarly, in another study (*21*), there was a general inhibition or reduction in chromosome breakage associated with medium in which fetal calf serum was used instead of horse serum. This is not to say that chromosome aneuploidy or mixoploidy *in vitro* is equivalent to neoplastic transformation, since all the mouse cell lines in the first study were mixoploid, whether neoplastic or not. In any case this alerts us to the existence of seemingly innocuous factors which may have important influences upon the genetic content of a line.

Thus the karyological monitoring of cell lines for "identification" is as important for the detection of evolutionary loss of identity as it is for the detection of mislabeling or the introduction of a foreign cell line by accident.[2]

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Identification Standards: Serology

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We can distinguish two phases in serological technology for identification of cell lines. During the first phase the objective is to determine the species of origin. Now, a more refined characterization of cell lines within species boundaries is desired. Various methods have been proposed which I will describe.

At a time when species determination was of greatest urgency, a straightforward approach led to a species-specific hemagglutination test (*1*). It was based on antigens common in cultured cells and on homologous erythrocytes. Antisera were produced against whole-cell homogenates. The resulting antibodies were predominantly species-specific in agglutinating red blood cells.

Such anti-cell sera were also used by Stulberg and co-workers (*2,3*) who tagged them with fluorescein and obtained species-specific fluorescence on homologous erythrocyte membranes. Furthermore, anticell sera exhibited species-specific cytotoxicity in the presence of complement, a method employed by Coriell's group (*4*).

Whereas the hemagglutination technique was not adaptable to differentiation of cell lines within species, this could be done with the Stulberg method (*5*). Labeled anticell sera were applied in different dilutions directly to cultured cells. High staining titers indicated close relationship. In a more elaborate modification, as used by Merchant *et al.* (*6*), anticell sera were cross-absorbed with cell lines under investigation, then labeled and applied in different dilutions directly to cultured cells. Significant differences are seen in staining titers dependent on whether related or unrelated cell lines were used for absorption. It is obvious that by this method cellular surface antigens are measured.

Coombs and co-workers took another approach with the mixed agglutination reaction (*7*). Again, they started out with antigens common to cells and erythrocytes and employed anti-red cell sera. These sera clumped homologous erythrocytes and cultured cells together. For species determination this test was advantageous, because the same reference serum could be applied to any cell line examined. However, the test sera had to be made specific by more or less complicated absorption procedures. To achieve species-specificity, cross-reacting heterophilic and blood group antibodies had to be removed. On the other hand, such heterophilic and blood group antigens in cells proved valuable for intraspecies differentiation (*8*). Here then, the species-specific and other cross-reacting antibodies were absorbed out. In my opinion, this method would be most suitable as a simple routine procedure if specific reference sera were made available from commercial or other central resources.

There is a major limitation, however, in both the immunofluorescence tests of Stulberg and the mixed agglutination reaction. Both procedures are based on the detection of cell surface antigens. The mixed agglutination test is even more limited in that it operates only with those surface antigens which are common in cells and erythrocytes. We have tried, therefore, to tackle the problem more comprehensively. Our goal is to analyze the overall spectrum of soluble and structural antigens throughout the cells. Let me outline briefly our progress.

Cultured cells were homogenized for injection into guinea pigs and other animals. Antisera were examined by double immunodiffusion against cell homogenates. We were surpised to find only a few precipitation lines with any of the sera, usually not more than two or three. However, some of the lines produced by one serum were often not identical with those produced by other sera. Studies on this phenomenon showed that individual variation of qualitative antibody response was due neither to chance nor to random antigenic competition, but was genetically determined (*9*). Inbred animals always produced identical antibody specificities which were characteristic for each inbred strain. When a variety of animal species was immunized against human cells, we found that some antibody specificities were formed predominantly, or even exclusively, in certain species (*10*). These observations largely explain why earlier investigators attributed poor immunogenic properties to antigens of tissues and cells.

In our analytical studies (*11*, *12*) we selected anticell sera that varied in antibody specificities and arranged them in such a way as to obtain clearly identifiable patterns with maximum numbers of nonidentical precipitation lines. We could distinguish 25 lines that were designated arbitrarily. Characterization of the antigens was aided by their differential susceptibility to heat, acetone, enzymes, and other factors (*13*). Some human cell lines were comparatively examined for the presence of those antigens. Differences were established with the exception of HeLa, Chang liver, and Chang conjunctiva (*11*).

Eight of the antigens were demonstrated on pooled human erythrocytes (11). These antigens seem to exist in dual form, *a*) as soluble entities which react in immunodiffusion, and *b*) as a structural part of cell and erythrocyte membranes.

It is hoped that these analytical data can be utilized for routine identification of cell lines. However, we expect immunofluorescence to be more suitable for such purpose than immunodiffusion. Purification and labeling of either the various antigens or the corresponding antibodies would be prerequisites. Together with K. Pomeroy, we have succeeded so far in preparing a pure antiserum against one antigenic component of human cells. In immunofluorescence of human cells, these antibodies stained a specific area which corresponds to the Golgi region. Apparently, a morphological parameter can be added to the parameter of serological specificity in the identification of cells.

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Biochemical Identification Standards—Discussion

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In my discussion of biochemical characteristics of continuously propagated cell lines, I propose to comment briefly and only on enzyme polymorphisms. The reasons for their use have been well introduced by Dr. Ruddle. The obvious beginning has been made by Dr. Stevenson and Dr. Coriell and concerns itself with the demonstration by Gartler (*1*) of the presence of the A isoenzyme form of glucose-6-phosphate dehydrogenase (G6PD) in all the heteroploid human cell lines he studied. His original observation has been extended and supported by a study initiated by the Cell Culture Collection Committee (*2*, *3*). As of now, 42 human cell lines, including most of the well-known ones, have been examined; all possess the A isoenzyme of G6PD, irrespective of the genetic background of the donors. In contrast, 26 human diploid and quasi-diploid cell strains from skin, lung, and blood have the B isoenzyme, in conformance with the genetic background of the donors. Insofar as we have been able to check, several heteroploid cell strains transformed by SV40 virus have been shown to retain the B isoenzyme found in uninoculated control cells.

These findings are significant in two ways.

First, in terms of the use of heteroploid cells for virus vaccine production, the problems of proving safety are placed at one level. For, if one considers these lines as they now exist as variants of HeLa, the variant with no tumorigenic potential must be selected. If one believes that these cell lines have resulted from an as yet undemonstrated mutational sequence, the same condition holds, since what other mutational events have occurred is not known.

Second, these findings provide impetus toward obtaining systematic genetic profiles of each cell line and strain. This may be expressed in part by enzyme polymorphisms. Additional dehydrogenases, kinases, transferases, and esterases, some of which have been discussed, can be used. By means of such a profile, changes in cells may be followed that include selective influences *in vitro*, as well as interspecific or intraspecific contamina-

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tions. Figure 1 illustrates a beginning isoenzyme profile, carried out on agarose. The arrow indicates the application line. The first two columns indicate the differing rates of migration of G6PD isoenzyme obtained from extracts of HeLa cells and Detroit-532, a diploid strain. The third, fourth, and fifth columns show lactic acid dehydrogenase (LDH) patterns obtained from Detroit-532, HEp-2, and HeLa. These patterns show that the diploid cell is deficient in LDH 1 and 2. The two heteroploid cells can be readily distinguished one from the other in the amounts of LDH 1 and 2 present. Other polymorphisms may reveal additional differences in completing such profiles.

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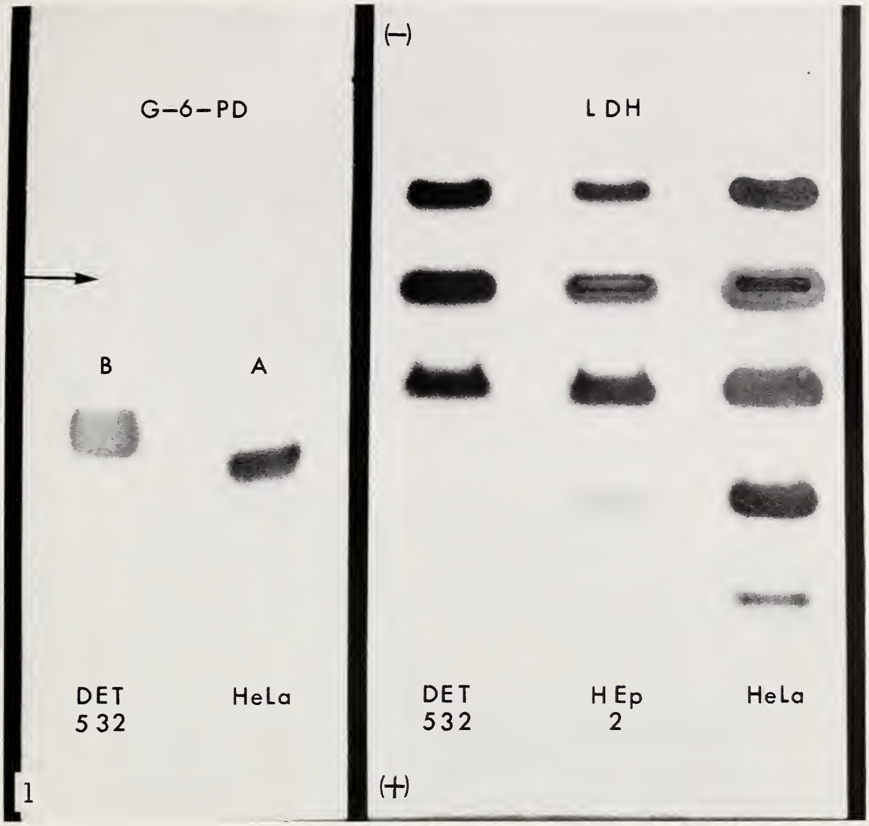

G–6–PD
B
A
DET 532
HeLa
1
(–)
LDH
DET 532
HEp 2
HeLa
(+)

FIGURE 1.—Representative G6PD and LDH zymograms of human cells.

GENERAL DISCUSSION

Dr. F. Deinhardt: I would like to make one comment stressing the instability of the karyogram of cell lines.

Dr. Marczynska, of our department, during the last 3 years has studied several cell lines which were all derived from one single nonhuman primate kidney obtained by unilateral nephrectomy. These cell lines were karyotyped after approximately every 5 cell culture passages. Eighty to ninety percent of the cells of some of these lines remained diploid for many generations, became heteroploid in further passages with less than 20% of the cells remaining diploid, and again shifted to diploidy (80% of the cells) after an additional 10 cell passages. Cells of some lines developed a specific marker chromosome which, however, was not stable and disappeared over 5–10 further cell passages, when another marker appeared. Some lines were heteroploid and still showed contact inhibition; others were diploid but had lost contact inhibition.

In summary, we have observed over the last 3 years, in these 8 or 9 cell lines all derived from one kidney, any combination of diploidy, heteroploidy, marker chromosomes, contact inhibition, and absence of contact inhibition. In addition, there was a considerable drift of these conditions during successive passages to the extent that a description of a cell line had also to be identified by the particular time of analysis, *i.e.*, a line shown to be diploid at a given passage level might have gone previously through a period of heteroploidy.

However, none of the cell lines, including a heteroploid cell line which also had lost contact inhibition and was morphologically altered, proved truly malignant in the strict sense of the word, *i.e.*, none produced tumors when inoculated into the same donor animal from which the original kidney was obtained.

Dr. D. Franks: When one does test for markers, whether they are antigens or isozymes, in cell cultures, it is important to realize that one frequently finds mixtures of two different types of cells. In several of our studies when we were looking for antigenic markers, we found that a culture of cells would contain both antigen-positive and antigen-negative cells (*Biol Rev* 43 : 1967).

In a clone of RK13 cells tested for the A antigen, there were some cells which were completely negative. I think this is a general finding, not only in our own laboratory but in several others. One has, therefore, to be very cautious about interpreting these sorts of tests as showing differences between cell lines.

For example, if one finds a particular antigenic marker in one cell line in a portion of the cells and then subsequently finds the culture is completely negative, does this mean that there has been contamination, or does it mean that the negative cells have overgrown the positive cells? At the present moment I don't think we know the answer to this.

A further point is that it is important to do tests which show the characteristics of individual cells. Interesting though isozyme tests are, what they show is the total isozyme pattern in the culture. One would like to know, for example, in these cultures which have the G6PD type-A isozyme, whether all the cells are producing type-A isozyme, or whether there is a minority of cells still in the culture producing a small amount of type-B G6PD which is detected by what is after all an insensitive technique.

Dr. G. Yerganian: Some 5 years ago, several sublines of a female Chinese hamster fibroblast-like cell line (FAF28), cloned 4 and 5 times previously to retain diploidy, were distributed to 2 very distant geographical points, Moscow and San Francisco. The parent line maintained in Boston underwent a rare cytological change which has occurred in about 1% of the cell lines thus far established in our laboratory.

The structural alteration was limited to the late-replicating segments of the X chromosomes, resulting in a tandem (inverted) duplication of one or both arms while autosomes remained intact.

Related events were reported by Zakharov *et al.* (*J Nat Cancer Inst* 33: 935–956, 1964) for the cloned diploid subline sent to Moscow. Recently, cytological check conducted in Boston on the clonal subline maintained by Dr. R. Painter in San Francisco revealed that the same mechanism leading to sex chromosome anomalies in the parent line had been involved in forming marker chromosomes. Thus diploid sublines derived from repeated clonings may undergo parallel alterations of the karyotype while maintained under different conditions at distant geographical points. This example is presented to point out the role of genetic determinants in patterning cytological trends in cultured cells.

The second point I wish to discuss is concerned with cell morphology and LDH isozyme banding patterns. A renal cell derivative (P3) of the Chinese hamster had all 5 isozyme bands. Well-differentiated tumors, resembling nephroblastomas, formed when P3 cells were implanted into related hosts. These tumors, like the parent epithelial-like cell culture, had all 5 isozyme bands. When tumors were cultured, fibroblast-like cells appeared; these were then cloned and later inoculated into animals. Cyclic explanting of tumor derivatives and clonal selection for the more slender versions of fibroblast-like cells resulted in a gradual reduction in differentiation or development of tubular elements in tumors, so that after 3 clonings tumors derived from fibroblast-like derivatives were fibrosarcomatous. With each cloning, the LDH isozyme banding pattern shifted stepwise from the parental 1–2–3–4–5 banding pattern to the 4–5 bands associated with the more slender fibroblast-like derivatives. It appears that the LDH isozyme banding pattern is closely associated with cell and tumor phenotype, thereby limiting its usefulness as a consistent phenotypic parameter for cultured cells.

Dr. K. G. Brand: I would like to make one comment concerning Dr. Moorhead's interesting finding that one contaminant apparently consisted of a mixture of mouse cells and chicken cells. There is one interesting phenomenon sometimes observed in transplanted mouse tumors—the so-called, double minute chromosomes which look very much like chromosomes of the chicken. This type of chromosomal aberration was recently described as a result of transplanting Rous sarcoma into mice. The authors discussed the possibility of hybridization of mouse and chicken material.

In our own studies on polymer carcinogenesis in mice where chicken material is certainly excluded, we see these double minutes occasionally.

Dr. J. H. Hanks: Studies on cell nutrition yield two bits of useful information. 1) a necessity for synthetic media is a prescribed obligation for studies of nutrition. 2) For other purposes one may well utilize a synthetic base, but one should not hesitate to supplement it with autoclavable sources of diverse nutritional factors.

As a matter of fact, Charity Waymouth, some years ago, demonstrated the nutritional usefulness of supplementation with bacteriological peptones. Her observations were with HeLa cells and with strain L fibrocytes. To generalize, 0.1–0.5% Bacto-peptone (Difco Laboratories, Detroit, Mich.) added to Eagle's medium lowered serum requirements by tenfold.

If one is concerned with production of cell crops and with replication of viruses, one must recognize that improvement of N nutrition reduces serum requirements. It is obvious that the highly standardized commercial peptones provide a cheap and appropriate way of obtaining this objective.

Dr. S. J. Pirt: I would like to disagree with some of what Dr. Hanks has said. If the bacteriologic peptones and proteoses are used as sources of nutrients, they can only supply amino acids and vitamins.

We know which amino acids and vitamins are required for the culture of animal cells, and I think it would be a backward step to go to these peptone sources instead of sticking to the defined mixtures of vitamins and the amino acids.

For the role of sera, the bacteriologic peptones will not be in fact substitutes for sera.

Dr. Hanks: I suppose these meetings are designed to illustrate the diversities of opinions. At one time I made a very careful study of this matter. This was perhaps in the years when the synthetic media were not so adequate as at present. But it is not at all difficult to show you experimental data illustrating the point that the serum requirement is very different in some of these synthetic media when you leave out or when you supplement these synthetic media with the bacteriologic peptones.

Oh, I see—maybe there is a point I didn't make clear. No one argues against the synthetic media. And in the experience which I describe, the synthetic media were allowed to remain—were kept as a constant, and to those, one added small levels of the bacteriologic peptones.

It is not the peptones versus synthetic media. It is peptones added to synthetic media.

Large-Scale Production of Indefinitely Propagated Cell Lines

Peter D. Cooper, *Department of Microbiology, John Curtin School of Medical Research, Australian National University, Canberra, Australia*

The idea that some animal cells can be handled just like other microorganisms is now a familiar one, and it was clear early that very large amounts could be grown. An incentive, of course, was to make virus vaccines. This objective is now achieved in the sense that at least one continuous cell line is currently in large-scale production for a commercially feasible purpose.

Unfortunately, such cells are very like cancer cells, and their theoretical but obvious risks are still preventing general use. Others at this Conference will discuss this handicap but I will not. Instead, I will summarize very briefly the many technical factors involved in bulk culture of continuous-line animal cells and their potential applications.

Animal cells that can be grown indefinitely differ from most bacteria only in that they are larger and heavier, physically and chemically more delicate, and have longer doubling times and more restricted growth needs. They also tend to stick to surfaces. Culture methods accordingly take account of these differences. I shall assume a general familiarity with such methods and concentrate on large-scale aspects under five headings:

1. Culture systems
   a) Monolayer culture
   b) Suspension culture
   c) Application of automation
   d) Comparison of monolayer and suspension methods
2. Medium preparation
3. Cell strains—derivation and maintenance
4. Contamination control
5. Production potential and usage
   a) Disadvantages and advantages of continuous cell lines
   b) How big a scale?
   c) Limitations and opportunities

CULTURE SYSTEMS

Monolayer Culture

Most metazoan cells in nature are attached to a surface, and monolayer culture is usually the easiest *in vitro*. The simplest large-scale method uses large individually sealed bottles that are slowly rotated. Such giant "roller tubes" give a most efficient yield of cells per liter of medium; the group at Plum Island Animal Disease Laboratory has scaled up this method most effectively, and Dr. Bachrach as a discussant will enlarge on this.

Monolayer culture is essentially a batch process; perhaps its main merit is simplicity and its main demerit is the skill and care needed to work sterilely on a very large scale.

Suspension Culture

The large-scale culture of bacteria, particularly the early chemostats and the later high-efficiency models developed at Porton, has greatly influenced work on animal cells. Continuous animal cell lines grow well if artificially kept in suspension, and the simple "stirred pot" is the easiest of several devices to scale up. In 1957 McLimans' group produced batch cultures up to 20 liters, and our own group, working in England in 1958, proved the feasibility of continuous-flow culture.

Continuously operated systems are either extremely simple or rather complex with as many safety and control devices as possible. We ran two machines for 4 years, each giving 10^9 cells per day in 1 liter; the longest run lasted 10 months and we used the chemostat principle with a turbidimetric device to prevent washout. Similar machines are now in use in several laboratories, all much more elegant than ours. In general, maximum cell concentrations are 2–3 × 10^6/ml and doubling times 16–30 hours; the "steady state" is difficult to achieve, conditions tending to "cycle." The cells prefer loy oxygen levels and I believe that Dr. Daniels as discussant will enlarge on this.

These systems developed in two directions.

First, Cohen and Eagle made a simple chemostat suitable for laboratory studies. With it others have now maintained steady states for up to 1 month, but these are unstable and conditions tend to oscillate if perturbed.

Second, more completely controlled, much larger stainless-steel assemblies (up to 100 l) are currently working at Pirbright in England for commercial scale production of baby hamster kidney (BHK) cells. Figure 1 is a photograph of the vessel and ancillary equipment. Rapid turnaround and reliable operation were obtained by coupling it permanently to the medium filtration unit and steam-sterilizing the lot *in situ*. A nontoxic antifoam enables air/CO_2 to be passed through the culture, giving responsive pH control. Some 120 × 10^9 cells can be made per week.

Application of Automation

There are many factors to control, and the steady state is easily perturbed; since the optimum growth ranges of *p*H, oxygen tension, and cell concentration are narrow, the system easily comes out of balance. Hence, automatic control is particularly desirable, and most culture factors (*e.g.*, oxygen and CO_2 contents, *p*H, temperature, medium and gas flow rates, cell densities, and glucose and lactic acid contents) have now been automatically controlled. So far, however, there is no system that will automatically adjust these factors to give a maximum growth rate.

Comparison of Monolayer and Suspension Methods

The suspension method is probably the more practical in a large scale, especially if infective materials are produced. However, it may not be the simplest to develop and the merits of each system must be examined.

MEDIUM PREPARATION

Animal cells need a complex, rather expensive and heat-labile medium containing salts, glucose or galactose, vitamins, amino acids, and almost always animal protein. Eagle's minimal medium gives a good balance, but for large-scale work, some components could be increased in concentration. Some nonphysiological components, such as carboxymethylcellulose, dispersing agents, and antifoams, not to mention antibiotics, are considerable aids to growth.

The medium should be completely defined, but the cells that are both useful and amenable all need serum, and we cannot yet replace it with defined components. Serum quality affects both reproducibility of culture and impurity of product.

The premixing of medium components as dry powders in a ball mill is a major step in cutting down costs and poor reproducibility. Such preparations are cheap to ship, quite stable, can be pretested extensively, and allow large-scale preparation.

Few components can be autoclaved, and most have to be filtered. Specially treated and prewashed cellulose-asbestos pads are used in a filter press, but the bacterial count before filtration must be under 10^3/ml.

CELL STRAINS—DERIVATION AND MAINTENANCE

Most large-scale studies naturally have used cells (*e.g.*, HeLa, L, or BHK) that grow easily. Fortunately, the BHK cell also gives a usable product (foot-and-mouth disease vaccine) and is not highly malignant. For most applications, however, suitable cell lines still need developing.

Cell lines should be cloned and the clones carefully selected for performance, *e.g.*, reproducibly rapid growth and high yields of products or low malignancy. This procedure may take months or even years. Once selected, their characters should be maintained by frozen storage and a careful scheme of limited subculture from the master seed, which itself must be carefully monitored for contaminants and malignancy.

CONTAMINATION CONTROL

Contamination control is essential for efficient production and for safety of the product. A routine testing laboratory is needed, especially for *Mycoplasma*. Antibiotics can and should be omitted from the master seed and the final brew to allow adequate testing, but could be used for intermediate steps and to obtain the initial clean cell line, which needs great attention. Use of closed systems (*e.g.*, completely piped) eliminates the main source of contamination, namely people.

PRODUCTION POTENTIAL AND USAGE

Disadvantages and Advantages of Continuous Cell Lines

As already mentioned, the main snag is the theoretical danger of malignancy. This might be negligible if the product is given to animals, or if it is purified, and purification is feasible on the large scales considered. Their advantages are: 1) independence of a regular supply of animals and relative cheapness, 2) greater or complete freedom from adventitious viruses, and 3) provision of a certified seed stock, thoroughly pretested over a period of years if necessary. These are solid advantages.

How Big a Scale?

The production potential is enormous in relation to demand. For example, a single 100 l fermentor could produce poliovirus at the rate of almost a million Salk-type doses, or 10–100 million Sabin-type doses per day, enough to satisfy world demand. Domestic animals need larger doses, and fermentors of 1000 l are projected. Running costs are low, and medium and serum supply could readily be geared up. Quality control is a big organizational problem, but such a large scale would permit concentration and purification of the product quite cheaply and would simplify quality evaluation as well as other problems.

Limitations and Opportunities

Oncogenic potential apart, the main restriction stems from the few suitable cell types and the poor definition of serum-containing media. More work is needed on these aspects. Production of multiple vaccines would be feasible if the scale-up in quality-control problems could be met. Although this is an expensive fermentation, cost is no handicap because of the very high value of the product, which may run as high as one million dollars a gram. Bulk fermentation could theoretically provide the crash production needed to counter a new epidemic of, say, influenza but suitable cell-virus systems are not yet to hand. Several other products (interferon, pure antibody, hormones) could in theory also be made in this way, but we do not yet know how to do it.

In summary, this method seems ready for a period of broadly based research and development.

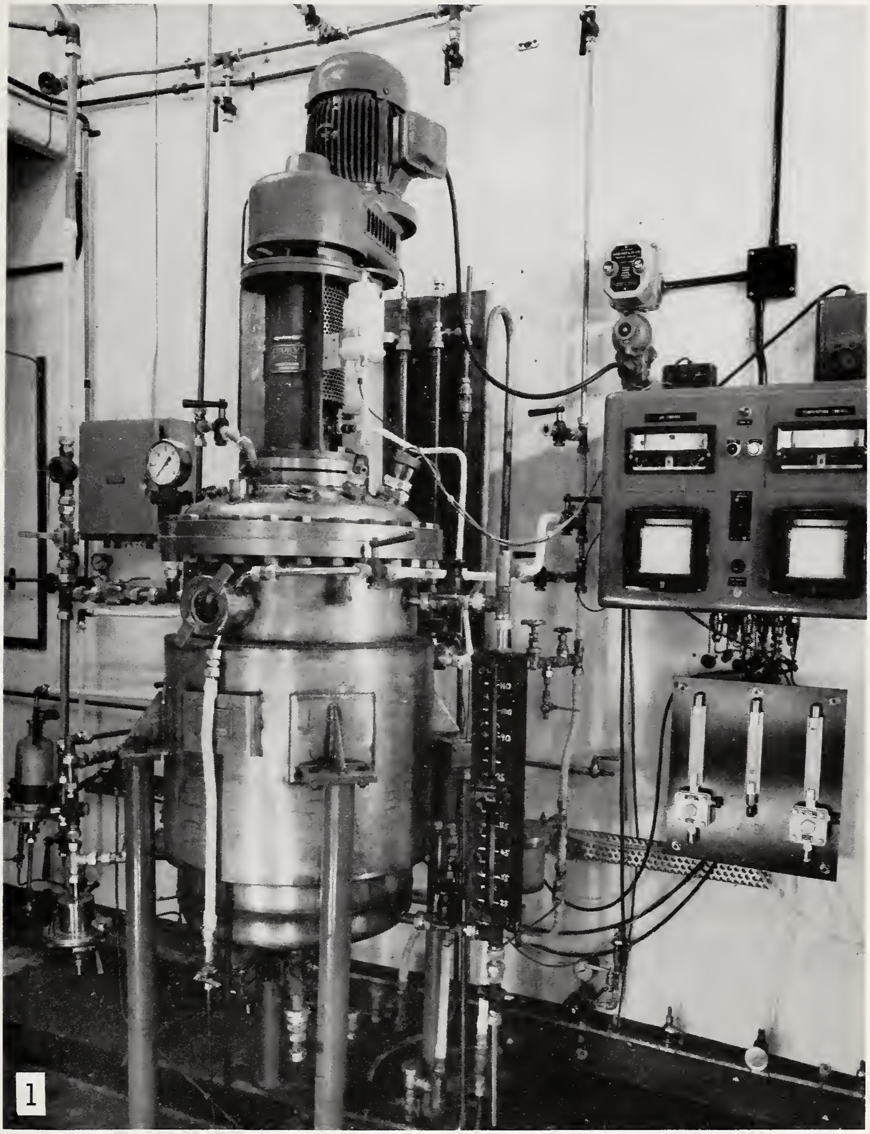


FIGURE 1.—Equipment used for large-scale growth of animal cells.
Courtesy of Dr. Telling, Animal Virus Disease Research Institute, Pirbright, England.

Comments on Technology of Production—Continuous Cell Lines

William F. Daniels, *Process Development Division, Department of the Army, Fort Detrick, Frederick, Maryland 21701*

I agree with Dr. Cooper that growth of cultures of established cell lines on a large scale has been shown feasible by many investigators. Monolayer cultures, however, are deceptively simple in that usually no control is available over environmental conditions, though McLimans and his workers (*1*) have accomplished this to an extent. Suspension cultures, on the other hand, can be achieved most economically and controlled in classic fermentors which can be scaled up through technology from microbial experience. Adequate technology for tissue culture medium makeup, transport, and sterilization is indeed at hand.

Two problem areas are: (*a*) maintenance of sterility in antibiotic-free systems and (*b*) maintenance of prescribed environmental conditions at all levels to minimize possible genetically selective forces.

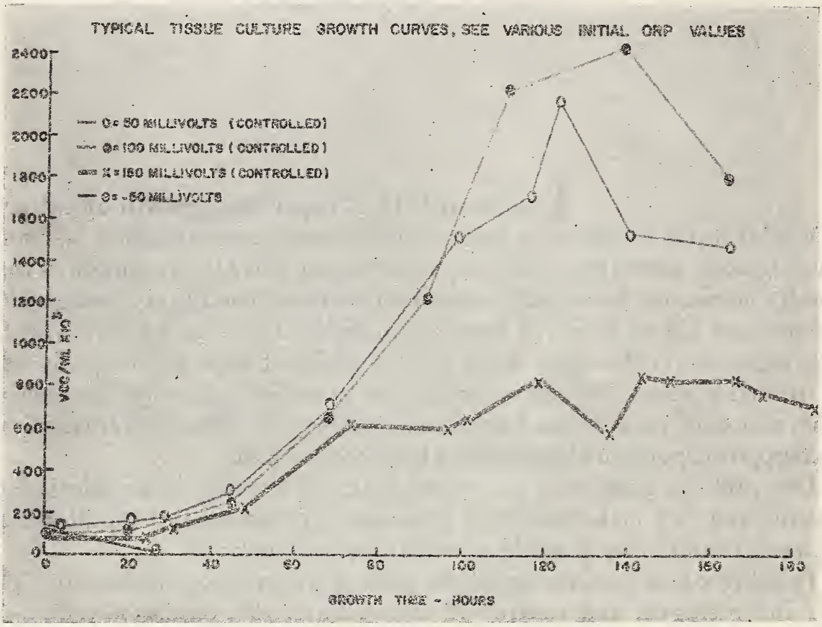
To achieve this involves agitation, gassing with proper mixtures of CO_2, air, and nitrogen, and control of temperature, *p*H, oxidation-reduction potential (ORP), and dissolved oxygen. Of these, *p*H, ORP, and dissolved oxygen are the most difficult to control. Until now, we have been reasonably successful with *p*H and ORP, but less so with dissolved oxygen. This has been due mainly to lack of a steam-sterilizable probe.

To date our investigations reveal control of *p*H and ORP to be of major significance, with dissolved oxygen of lesser value, though related to ORP values.

We find rapid changes, or the exceeding of certain limits of *p*H, to restrict rates of growth in rapidly growing cultures. Secondly, if gas mixtures are not used, then the total amount of bicarbonate needed for *p*H control can become growth limiting.

ORP measurement and control are of importance in tissue culture in two areas: (*a*) quality of medium—our data reveal a significant relationship between growth rates and ORP levels in the same medium—and (*b*) suspension cultures—to insure similar environments, the ORP level is an important consideration to permit equating conditions between vessels of different geometric configurations.

Text-figure 1 shows the growth curve response of Earle's strain L cells in suspension culture at different constant ORP levels maintained during the entire growth cycle. While ultimately, varying levels at certain points in the growth cycle may be desirable, these data do show that an optimum growth response must exist for a constant ORP level. Sparged air is used to restore the desired level which has been lowered by the reducing activities of growing cells. Thus, the quantity of air delivered is in response to the actual metabolic activities and growth of the cells.



Text-figure 1.—Growth curves of Earle's strain L cells at different controlled oxidation-reduction potentials.

Instances have been noted where medium not properly poised has destroyed cells, yet, when properly poised, supported excellent growth.

From this work, we conclude that there are no real technical obstacles to prevent growth of established cell lines or other types, for production of vaccines in suspension cultures. That this is true is borne out by the recent report by Weiss and Schleicher (*2*) who have modified a suspension system to afford growth of several cell types including WI–38.

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Large-Scale Cultivation of Animal Cells in Roller Bottles for Production of Decigram Amounts of Pure Foot-and-Mouth Disease Virus

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THE purpose of this report is to review procedures in use at our laboratory for 1) the large-scale production of cell cultures on the surfaces of rotating 2-liter Baxter bottles and 2) the use of such cultures for producing large amounts of purified foot-and-mouth disease virus (FMDV) (*1–4*).

MATERIALS AND METHODS

The culture bottles are held in 19-place racks (fig. 1) which are rotated at 2 rph on a 3-tiered roller mill (fig. 2) that accommodates over 2,000 bottles. The racks are made in 2 sections which are joined together by 3 wing nuts after the bottles have been placed in them. This construction holds the bottles within the racks during their inversion for emptying (fig. 3). Instead of stoppers, a sheet of polyurethane foam bonded to the underside of the lid compresses a sheet of sterile aluminum foil tightly against the lip of each bottle. Bottles are removed from the racks only for replacement due to accidental breakage. They are washed, dry-heat sterilized, seeded with cells, infected and harvested of virus, and decontaminated with acetic acid while situated in the racks. The racks containing the bottles fit directly onto the headers of a glassware washer (fig. 4).

Details of cell growth and of virus growth and purification have been described previously (*1–4*). Primary calf-kidney (CK) cells and baby hamster kidney (BHK) cells, passage 21, have been grown in the bottles. Growth medium for the BHK cells consists of 80% modified Eagle's medium, 10% tryptose phosphate broth, and 10% bovine serum buffered at

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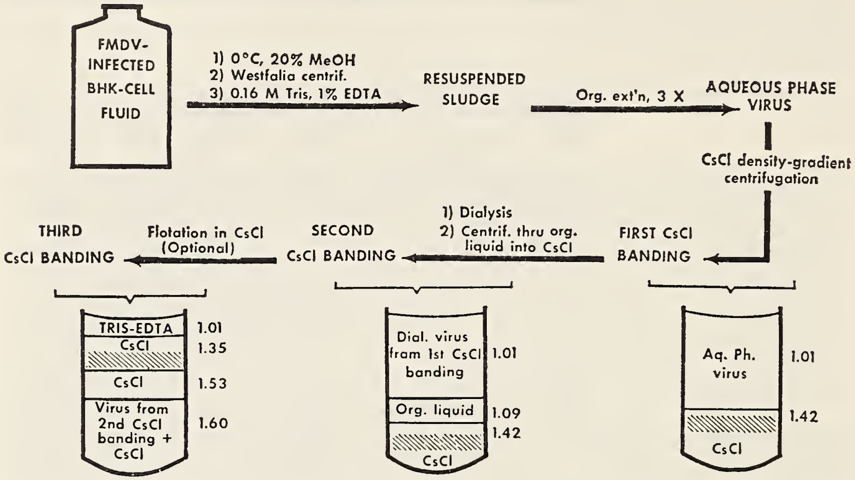
[1] U.S. Department of Agriculture.

*p*H 7.5 with 0.02 M Tris(hydroxymethyl)aminomethane. Most production has been of FMDV, type A, strain 119 in BHK cells. The virus is concentrated and purified by methanol precipitation, organic solvent extraction, and up to 3 different bandings in cesium chloride density gradients (text-fig. 1).

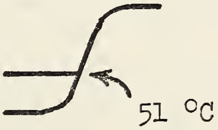

FMDV-INFECTED BHK-CELL FLUID
1) 0°C, 20% MeOH
2) Westfalia centrif.
3) 0.16 M Tris, 1% EDTA
RESUSPENDED SLUDGE
Org. ext'n, 3 X
AQUEOUS PHASE VIRUS
CsCl density-gradient centrifugation
FIRST CsCl BANDING
1) Dialysis
2) Centrif. thru org. liquid into CsCl
SECOND CsCl BANDING
Flotation in CsCl (Optional)
THIRD CsCl BANDING
TRIS-EDTA
1.01
CsCl
1.35
CsCl
1.53
Virus from 2nd CsCl banding + CsCl
1.60
Dial. virus from 1st CsCl banding
1.01
Org. liquid
1.09
1.42
CsCl
Aq. Ph. virus
1.01
1.42
CsCl

TEXT-FIGURE 1.—Flow diagram for purifying FMDV from cell fluids in 5 steps—methanol precipitation, extraction with organic liquids, and up to 3 different kinds of bandings in CsCl density gradients. Conditions are shown for the initial loading of the centrifuge tubes, including specific gravities of the liquids. The final light-scattering bands of virus are depicted by *diagonal lines* at the position of viral isodensity, 1.43 g/ml.

RESULTS

Only one-fifth of the 2,000-bottle capacity of the roller mill has been used to date. Primary CK cells grow out to monolayers containing 100 million cells and BHK cells to multilayered sheets containing 800 million cells.

Infectious fluid from BHK cultures contains about 5×10^8 plaque-forming units (PFU)/ml. The overall recovery of infectivity during concentration and purification is about 33%. From each rack, 6 mg of purified virus is obtained, and a total of 110 mg is produced each week. The purified virus is present in solution at a concentration of 5 mg/ml and an infectivity of about 5×10^{11} PFU. The virus is homogeneous by the biological, chemical, and physical tests shown in text-figure 2, *i.e.*, by ultracentrifugal, electrophoretic, isopyknotic, specific-infectivity, absorbance-temperature, protein-to-RNA ratio, and base composition measurements, as well as from measurements of its amino acid composition and C-terminal sequences (*1*, *5–8*).

Parameter		Values
Size (diameter)		23 mμ
Sedimentation constant		140 Svedbergs
Electrophoretic mobility		2.92×10^{-5} cm^2/V·sec
Isodensity in CsCl		1.43 g/ml
Specific infectivity		10^{14} PFU/g
Virion:PFU ratio		~10^3
A-T profile		
Protein:RNA ratio		69:31
1% Extinction coefficients	Virus	76.0 at 259 mμ
	RNA	220 at 258 mμ
	Protein	11.1 at 276 mμ
Base composition		.24G/.26A/.28C/.22U
Amino acid composition		Constant
C-terminal sequence		---SER-ALA-LEU-GLN

TEXT-FIGURE 2.—Parameters of homogeneous foot-and-mouth disease virus, type A_{119}.

DISCUSSION

Large-scale culturing of cells on glass in rotating Baxter bottles has the practical advantage of being applicable to the growth of primary as well as tumorigenic cells. While the potential capacity described above is 2,000 rolling bottles, the method has been scaled-up elsewhere to about 28,000 bottles (*9*, *10*). Animal and human viruses produced in primary cells have been utilized in vaccines in the United States after appropriate potency and innocuity testing, whereas viral vaccines derived from tumorigenic cells, such as the BHK-21 cell line, have not been approved. This restriction, at least for cell-free vaccines, may now be reconsidered, since evidence from several laboratories (*11*) indicates that tumors can be produced in Syrian hamsters only by BHK-21 cells which are intact and viable. In addition, when viruses have been purified and characterized to the extent described for FMDV, they should be free of extraneous toxic, anaphylactic, or oncogenic activities regardless of their cellular origin. Finally, the pure FMDV described herein is a potent antigen. As little as 0.16 μg which has been inactivated with acetylethyleneimine and mixed with incomplete Freund's adjuvant stimulates the production of neutralizing antibodies in guinea pigs (*12*).

SUMMARY

Baby hamster kidney (BHK) cells (as well as primary calf kidney cells) are used to produce large quantities of foot-and-mouth disease virus. Two-liter Baxter bottles seeded with BHK cells in a Tris(hydroxymethyl) aminomethane-buffered growth medium are placed inside 19-place cylindrical wire racks and rotated on a 3-tiered roller mill. The bottles are not removed from the racks at any time during their use. After 6 days, each BHK culture bottle contains a multilayered sheet of about 800 million cells. Infection of these sheets with virus yields viral progeny at a concentration of about $10^{8.7}$ plaque-forming units (PFU)/ml. Virus is concentrated and purified by methanol precipitation, extraction with organic solvents, and density-gradient centrifugation. Recovery of infectivity is about 33%. From each rack, 6 mg of purified virus is obtained, and a total of 110 mg is produced each week. The virus is present in solution at a concentration of 5 mg/ml and an infectivity of about 5×10^{11} PFU. The virus is homogeneous by several biological, chemical, and physical measurements.

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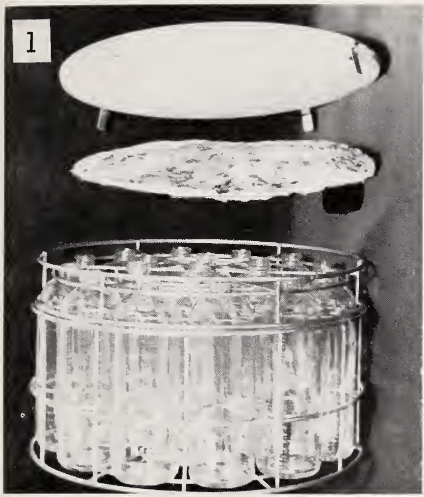

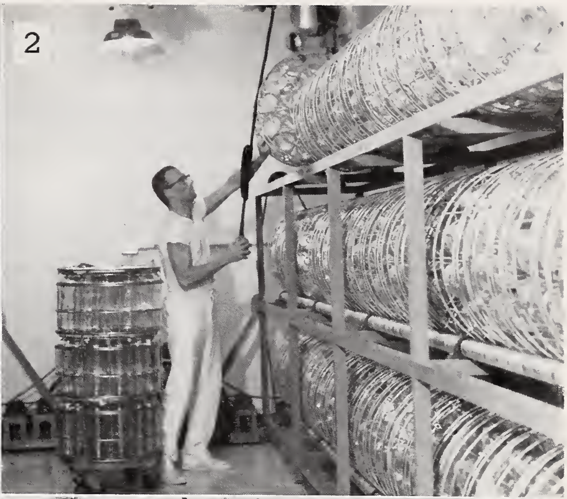

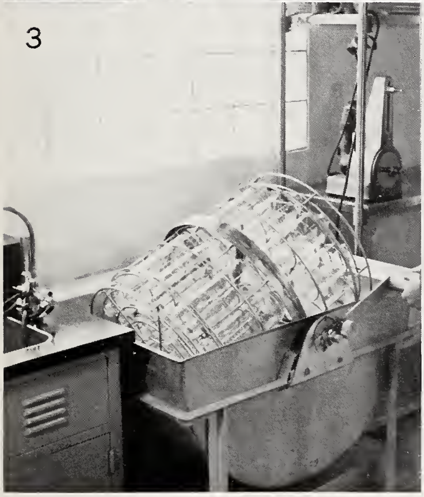

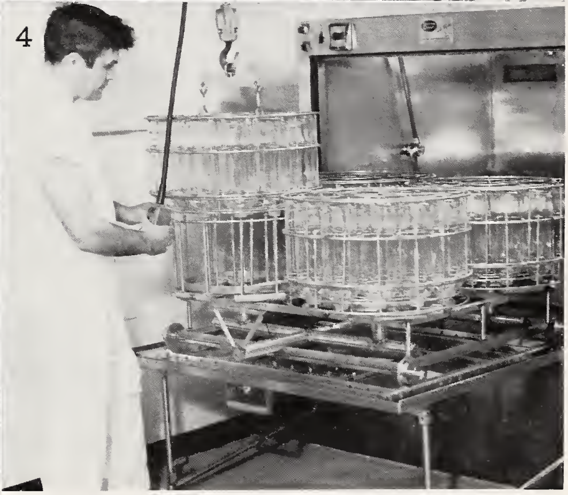

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Figure 1.—Cylindrical rack for holding nineteen 2-liter Baxter bottles. Polyurethane foam bonded to the metal lid compresses an aluminum foil sheet against the lip of each bottle.

Figure 2.—Three-tiered roller mill for rotating the racks. Rotation rates for attachment and growth of cells are 3 rpm and 2 rph, respectively.

Figure 3.—Two-place inverter for discarding spent media and collecting virus fluids.

Figure 4.—Glassware washer which accommodates 4 racks of Baxter bottles.

GENERAL DISCUSSION

Dr. F. Rapp: The criteria given by Dr. Bachrach for purified virus would be insufficient to detect certain types of contaminants. This is especially true for foreign determinants being carried in the capsid of unrelated carrier viruses, a phenomenon we will be discussing later. The usual biophysical techniques being used to purify viruses are not entirely sufficient to rule out such contamination.

Dr. J. Warren: I don't want to deprecate the elegant engineering and technology we have been hearing about from Dr. Bachrach and Dr. Holper. But it seems to me that we should think about applying these to specific vaccines. At the present time we are confronted with the possibility of preparing vaccines which will have relatively long incubation periods, with the possible exception of poliomyelitis vaccine where we may have to find a substitute for primary monkey kidney. If, then, we are thinking of the vaccines of the future, as for rubella, which is a very slow virus, if we are thinking of a vaccine or vaccines in the field of tumors—and I'm being deliberately vague—which are all relatively slow to reproduce then I don't see how we are going to use any techniques such as Dr. Bachrach has used for foot-and-mouth virus (which grows in about 18–24 hours) for agents which will have to be fed and watered in culture cells continuously for a week or more. I think that we have to bear in mind that the problems in making large batches of purified virus multiply a thousand-fold with the length of incubation period of that particular agent.

Dr. J. C. Holper: I don't want to get into an argument with Dr. Warren, but I would point out that with rhinoviruses and human diploid fibroblasts we have been able to propagate rhinoviruses to much higher titers in the roller system similar to what Dr. Bachrach has described—not nearly as fancy I'm afraid—with good success.

One of the interesting by-products of this was that the multiplication phase of the rhinoviruses was much reduced in time over what was required with stationary cultures. So that cytopathology, for example, was evident in 24–48 hours as opposed to that of the stationary culture requiring 3–4 days.

Dr. H. L. Bachrach: A biological indication of the purity of FMDV is obtained from specific infectivity and physical particle/PFU ratio measurements. The specific infectivity of purified virus is 10^{14} PFU, and the virion/PFU ratio is approximately 10^3.

Putting these two values together, you arrive at 10^{17} virus particles per gram, which is the theoretical value for pure FMDV.

Suspension cultures are limited to tumorigenic cells and are lost entirely in case of bacterial contamination. With roller bottles one can also increase the concentration of virus at harvest by using a small amount of fluid during infection.

In order to avoid the inoculation of nonviral components into man and animals, more consideration should be given to the use of purified virus in vaccines.

Dr. P. D. Cooper: I would just like to make one point about the purification following on a point which was made from the audience.

In order to purify a product, it is better to purify it from specific contaminants rather than simply to constant properties of the preparation alone. This means that in our particular case we have to know what we are purifying the product from.

Cell Substrates for Human Virus Vaccine Preparation—General Comments[1]

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ONE of the major tenets of modern cytogenetics is that the cells composing a primary tissue culture precisely reflect the chromosomal constitution of those cells forming the tissue of origin. This premise, upon which rests the many significant developments made in the past few years in mammalian cytogenetics, is as well established as is our expectation that the results of tests made, for example, on blood samples or tissue biopsies are a faithful representation of the biology of the tissue sampled. Consequently, it should be readily apparent that primary cell cultures, if they are made from normal tissue, are composed of diploid cells. Since all cell populations currently licensed for use as substrates for vaccine preparation must be primary, they are all presumably diploid. Thus, diploid cells *per se* cannot have attributes that would preclude them from being used as a substrate for human virus vaccines. On the contrary, this is a desirable property of any cell system used for virus vaccine preparation. The point at issue is, therefore, *not* the question of cell ploidy but the effect of serial subcultivation on the biology of cells that divide *in vitro.*

IDENTITY OF PRIMARY CULTURES AND DIPLOID CELL LINES (STRAINS)

Primary cultures were first chosen as the only suitable substrate for vaccine preparation at a time when it was believed that the serial propagation of cell populations inevitably led to the acquisition of undesirable properties. This decision was based on an erroneous extrapolation of experiences with mouse cells which are uniquely unstable when cultivated *in vitro.* It is now well recognized that the cells of man and many other

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animal species can be serially propagated with full retention of the properties of the cells composing the tissue of origin. Most importantly, unlike mouse cells, the cells of man and of other animals do not inevitably acquire malignant or other abnormal properties. It is of the greatest importance, therefore, to appreciate that, other than operationally, there are no important, measurable biological parameters that distinguish a serially passaged unaltered (untransformed) cell population from a corresponding primary cell culture. The generally accepted definition of a primary culture is a cell population derived from animal tissue, cultivated *in vitro*, and which has undergone no subcultivations (*1*). Once subcultivated, unaltered cell populations derived from normal tissue can be referred to as cell lines[3] (strains) and, like primary cultures, are diploid (*2*).

Depending on the density of the initial inoculum, it is important to observe that the cells in primary cultures may undergo many *cell doublings* before use as a vaccine substrate. A primary culture begun with a few cells will undergo many more cell doublings before becoming confluent than a similar culture initiated, for example, with half of the total cell capacity of the culture vessel. Since primary cultures are acceptable as human virus vaccine substrates after many cell doublings, serially passaged diploid cell populations should be equally acceptable. Trypsinization cannot be invoked as a procedure that would necessarily give rise to an unacceptable serially propagated cell population, since it is universally used in the production of primary cultures now used for vaccine production. These concepts are illustrated diagrammatically in text-figure 1.

If the most important single requirement for the acceptance of cells for human vaccine preparation is their identity with "normal" cells, then both primary and unaltered diploid cell populations qualify. Antithetically, primary mixoploid cells or cells that have undergone a spontaneous or virus-induced alteration *in vitro* are unacceptable (*3–6*). Thus, deviations from the normal karyotype should be sufficient cause to exclude any cell population from use in preparation of human virus vaccines. For this reason alone, those cell populations with unlimited division potential defined by us previously as (altered) cell lines should be excluded from consideration (*2, 7*). Surely, if one is given the choice of producing a vaccine in a cell population with abnormal properties or in one with normal properties, the decision is clear. This is especially so since abnormal heteroploid cell populations offer no compelling advantages over use of a normal unaltered diploid cell population.

Thus, currently licensed primary cultures are diploid as are passaged diploid cell populations, and primary cell populations undergo numerous *cell doublings* before use, just as do the passaged diploid cell populations. The only difference between the two populations is that, unlike the primary culture, a passaged diploid cell population has been subcultivated from one vessel to another one or more times. Yet, in spite of this, a passaged diploid

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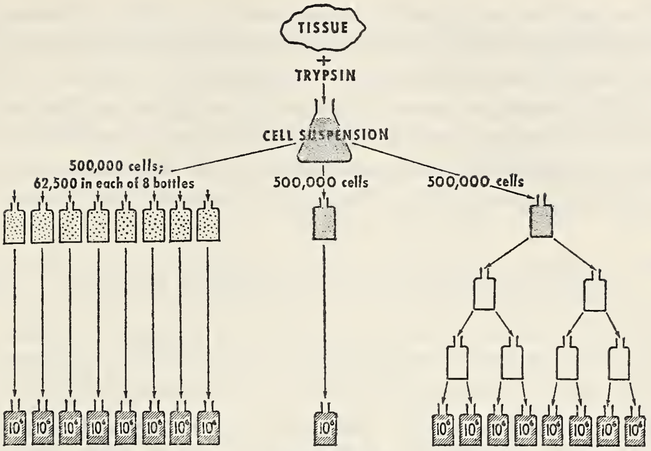
[3] Following the terminology required in the Appendix of this monograph.



	PRIMARY	PRIMARY	CELL STRAIN
Ploidy	DIPLOID	DIPLOID	DIPLOID
Subcultivations	0	0	3
Cell doublings	4	1	4
Days to obtain net cell yield	7	3	9
Net cell yield	7,500,000	500,000	7,500,000

TEXT-FIGURE 1.—Depicted here is a diagram of 3 possible ways in which cell cultures can be prepared as substrates for vaccine preparation; 500,000 cells are used to initiate cultures in the 3 examples and total capacity of any single culture vessel is assumed to be one million cells. Example on *left* assumes distribution of the 500,000 cells to each of 8 vessels. Example in *center* has 500,000 cells introduced into a single vessel. Although both cultures are diploid and primary, 15 times as many cells are produced in the multigeneration example to the *left* as are produced in *central* example. Despite this, both represent primary cultures. On *right*, a cell population is illustrated differing from the 2 primary examples only operationally, in that the cells have been subcultivated 3 times. Thus, all 3 examples are diploid and all are cell populations that have undergone one or more doublings. Despite the arithmetical and biological identity of the 3 cell populations, only the *left* and *central* examples are the types currently acceptable as substrates for virus vaccine production.

cell population with proper attention to arithmetic may have undergone as many or even fewer *cell doublings* than a lightly seeded multigeneration primary culture. Apparently, a multigeneration culture could be one that has undergone no subcultivations, one subcultivation, or many subcultivations. If it undergoes no subcultivations, we can call it primary. If it undergoes one or more subcultivations, we call it a passaged diploid cell population. Yet both are multigeneration cultures. This, then, is the kernel of the problem. Certainly no biological event occurs that can distinguish a primary culture from a passaged diploid cell population. They simply cannot be distinguished.

Thus we find ourselves in the awkward position of being incapable of distinguishing primary cultures from passaged diploid cell populations, yet permitting one to be acceptable as a vaccine substrate and the other not. Ironically, human virus vaccines are currently being licensed for distribution after being produced in cells with the very properties that are thought to be expressly forbidden. The astute vaccine producer can double his yield of legal vaccine merely by halving his primary cell inoculation density.

ADVANTAGES OF PASSAGED DIPLOID CELLS

If we are correct in our deduction that passaged diploid cells are, like primary cultures, both diploid and multigeneration, then what advantages are to be gained if we use these cells in human virus vaccine production? In a single word the answer is "standardization." A single tissue from a single donor has the potential of yielding great quantities of a diploid cell population which can be preserved at low temperatures and then exhaustively examined for safety *before* use. This maneuver makes it possible to provide a uniform, fully characterized stock of seed cells available for the production of a wide variety of viral vaccines. Conceptually, this approach is identical to the "seed virus" system currently employed in vaccine production, in which a seed virus is fully characterized and then used to initiate all vaccine production, thereby assuring product uniformity. In a similar manner, a "seed cell" system is possible with passaged diploid cell populations because specific tests can be introduced to insure that the cells used for vaccine production do not differ in any way from the "seed cells" or even from primary cultures.

It would appear then that, if the same principles for the control of the viruses used in vaccine manufacture were to be applied to the control of the cells used for their propagation, many of the existing problems with primary cell cultures could be circumvented. Paramount among these problems is the question of latent virus contamination of primary cultures, the risk to laboratory personnel of a fatal B virus infection, or infection with that mysterious agent from green monkeys that recently killed seven people in Germany, to say nothing of the potential hazard of the hybridization of the vaccine virus with an occult viral genome that may be present in primary cells (*3*, *4*, *6*, *7*). Such latent virus infections still can escape detection by current safety tests simply because the primary cell populations used are not subcultivated until the termination of their finite *in vitro* lifetime and are not scrupulously tested throughout this period. Such tests can and have been performed by many different laboratories on human diploid cells throughout their *in vitro* lifetime and before use as a vaccine substrate. Thus, from a single, carefully examined cell population, large quantities of a number of vaccines could be produced by several production facilities from a standard cell population derived from the tissue of a single donor. Modern cell culture techniques make it possible to derive such a standard diploid cell strain by cloning from a single cell, if necessary, to insure the ultimate in uniformity (*2*). This is similar to the plaque procedures used for purifying virus populations. Current practice, in which many new primary cell populations are continuously derived from vaccine production, repeatedly subjects vaccines produced therein to the inherent risks of latent virus contamination. The laudable concept of the "seed virus" now generally in use was invoked for the identical reasons of reducing the risk of change in biological materials.

Currently, human virus vaccines are largely produced in monkey kidney and chick embryo cells, two animal species representing examples *par excellence* not only of reservoirs for latent viruses but of the most serious kind, the oncogenic virus. The use of hundreds of primary dog kidneys represents yet another tissue source in which our ignorance of the kind and frequency of latent virus content is virtually encyclopedic. Yet, in spite of this, vaccines produced from dog kidneys were licensed *after* the recognition of many latent viruses in monkey kidney and, in particular, of the oncogenic virus SV40. Passaged human diploid cell populations derived from embryonic lung, on the other hand, have not been found to harbor latent viruses. Even if this happened occasionally, the fact that a passaged human diploid cell population could be exhaustively tested *before* use as a vaccine substrate is a crucial consideration. However, numerous passaged human diploid cell populations that have been examined have not revealed latent viruses. The human diploid cell population WI-38 has been more thoroughly tested by more laboratories throughout the world for latent viruses than any primary tissue. Unlike primary tissue, no latent viruses have ever been found. The choice of embryonic human cells is based on the general belief that embryonic tissue is less likely to harbor latent viruses than the cells of adult animals. Embryonic human cells also have the advantage of a much greater doubling potential than the cells of adult humans and thus, a longer, useful lifespan (*8*, *9*). Another advantage in the use of human embryonic fibroblasts is that spontaneous alterations or transformations are not known to occur (*8*, *9*). Spontaneous alteration of monkey kidney cells is frequent, and the number of potential cell doublings of chick cells is far too low to make them useful as a passaged diploid cell population.

ADVANTAGES OF PASSAGED HUMAN DIPLOID CELL POPULATIONS

The use of human cells for vaccine production has sometimes been criticized on the basis that any potential human cancer virus would be more likely to replicate in human cells than in the cells of other species. Thus, it is concluded that a greater risk is entailed in use of human cells than the cells of other species. However, Rous sarcoma virus, polyoma, SV40, and the oncogenic adenoviruses are all known to cross the species barrier and to replicate in the cells of animal species other than those to which the virus is indigenous. Indeed, the only known primate oncogenic viruses, SV40 and the oncogenic adenoviruses, are *not* known to be oncogenic *in vitro* or *in vivo* to the cells of the species in which they are indigenous, but express their oncogenicity in the cells of other species. One is, therefore, forced to the opposite conclusion that, from this standpoint of safety, the use of a vaccine prepared in cells of the homologous species to which a vaccine is to be administered is potentially the safest.

Furthermore, for the argument to be valid, that serially propagated human embryonic cells may harbor an unrecognized virus pathogenic for man, such a virus must 1) be able to cross the placenta, 2) remain latent in culture and produce no karyologic, cultural, or morphologic changes during many serial passages, 3) produce no changes in a wide variety of other cell types, both human and animal, 4) produce no effects in many species of experimental animals, and 5) propagate in culture to the extent that it is not lost by dilution during 30 or 40 cell generations. Taken together, this highly unlikely combination of circumstances should be weighed against the *known* difficulties inherent in primary monkey kidney and chick embryo cells.

A third advantage in use of human cells for vaccine production is that these cells have the broadest human virus spectrum of any tissue culture system known. Those of us who are interested in potential rhinovirus vaccines are faced with the certain prospect of having to use human cells, since many rhinoviruses are uncultivable in the cells of other species.

VACCINE TRIALS IN HUMAN DIPLOID CELLS

In recent years, over one million people have received trial vaccines produced in the human diploid cell line WI–38 to such virus diseases as those caused by poliomyelitis (*6*, *10–18*), adenovirus type 4 (*19–21*), rhinoviruses (*22*, *23*), and rubella (*24*). No untoward results have been observed. Oral poliomyelitis vaccines produced in WI–38 were first made and administered in the United States (*6*) and then in Europe (*11–14*, *17*, *18*) to a total of over 400,000 children. This vaccine has been found to be efficacious and to produce no untoward results. An adenovirus type 4 vaccine, also produced in WI–38 and administered in enteric-coated capsules, has now been fed to 600,000 men from 1965–67 in recruit training centers in the United States (*19–21*).[4, 5] No untoward effects have been observed and a significant reduction in respiratory infection due to adenovirus type 4 has been observed. As reported at this Conference about 30,000 persons have received parenterally administered measles vaccine

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[4] Personal communication from E. L. Buescher, Col., MC, Principal Investigator, USA Med R & D Command Project 3A014501B71Q, Communicable Diseases and Immunology, Task 01, Work Unit 166, Viral Infections of Man. Harry M. Rose, M.D., collaborated in this work.

[5] This vaccine field trial was sponsored by the Naval Medical Field Research Laboratory, Captain James H. Boyers, Commanding Officer, and is the result of a collaborative effort between the Bureau of Medicine and Surgery, U.S. Navy, and the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland.

prepared in WI–38 (*14*, *25*). This vaccine has been found to be efficacious and to produce no untoward effects in the recipients. There are also published reports dealing with the potential use of human diploid cells in the preparation of such vaccines as rabies (*26*), vaccinia (*27*), varicella (*28*), and arboviruses (*29*). Minimum requirements for the production of virus vaccines in passaged human diploid cell populations have now been established by interested groups of investigators, and these have been adhered to in the production of vaccines referred to above (*30*, *31*).

Finally, as Dr. Murray pointed out earlier, at the request of Dr. Shannon, the Director of the National Institutes of Health, a Task Force on Tissue Culture Viruses and Vaccines was established in 1962 to review knowledge on the precise topic of this Conference. This report, published in 1963 (*32*), contained suggestions on what sequential steps should be taken to demonstrate that a continuously propagable cell population was satisfactory for general production and use. After trials performed in small numbers of volunteers it was concluded: "If all has gone well, the fourth step might consist of a field trial of the vaccine involving several thousand persons. If the results continue to be favorable, the next step might be a larger scale field trial as a prerequisite to approval for general production and use." I would suggest, therefore, that WI–38 has met these and other criteria for acceptability detailed in the report to Dr. Shannon. In view of the several additional serious risks of using primary populations realized since that report was written and culminating in the present serious situation where vaccines produced from green monkey kidneys are not being licensed in this country, I am hopeful that a new impetus will be given to these considerations—especially since, in the same period, only favorable experiences have resulted from the use in one million people of vaccines produced in the passaged human diploid cell population WI–38.

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Tissue Cell Cultivation—General Comments

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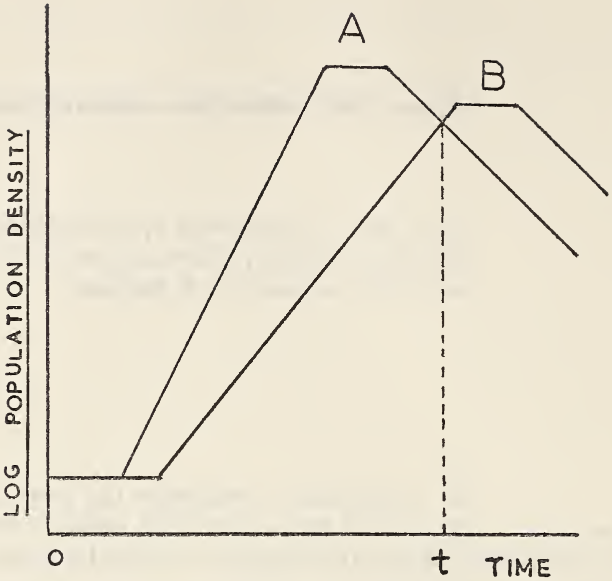
I would like to emphasize the need for a more analytical approach to growth of tissue cells. The relations between environmental conditions and growth parameters, such as lag period, population doubling time, and maximum population density, are vague and unpredictable. If one compares 2 growth conditions A and B (text-fig. 1), the basic differences between cultures A and B are in the 3 growth parameters lag, population doubling time, or specific growth rate and maximum population density. It is rare, however, to find any analysis of cell growth in terms of these basic parameters. Often one is only told the population obtained after a given time, sometimes even with an unspecified number of medium changes. If the criterion of "best growth" is population after a given time, in the case shown in text-figure 1, it is crucial to know whether the time is before or after *t*. Analysis in terms of the growth parameters will also point out new information about the role of various nutrients in cell growth and function. For instance, it may show that the role of some nutrients is confined to shortening the lag period. The analysis also will provide the quantitative basis of tissue cell culture which is essential for applications such as virus production.

GROWTH-LIMITING FACTORS

One of the most important problems is what limits the maximum population density. The population density can be limited by 3 factors only: (1) exhaustion of an essential nutrient; (2) some inhibitory condition such as an adverse *p*H value or an inhibitory product, and (3) the lack of physical space. The latter can hardly be of importance in a suspension culture. Usually, in suspension cultures, the maximum cell population attained without any change of medium is less than 2 million cells/ml, *i.e.*, a cell dry weight of about 1 mg/ml. Whether the factor limiting growth to this



Text-figure 1.—The significance of the basic growth parameters are shown by two idealized growth curves (A and B) which differ in their lag periods, population doubling times, and maximum population densities but have the same durations of maximum population density and the same rates of population decay.

level is an inhibitor or a nutrient exhaustion is a question which has not been answered with certainty, although speculation seems to have favored the inhibitor hypothesis. The nutrient exhaustion hypothesis could be tested if the growth yields were known: for example, how many or what mass of cells can be obtained from a given amount of a nutrient when there is no inhibitory factor in the medium? Values for growth yields in tissue cultures are, in fact, nonexistent in the literature. In one of the basic papers on the amino acid nutrition of cells Eagle (*1*) tells us the amount of amino acid required to sustain growth of HeLa cells at the maximum rate in monolayers if one changes the medium every day. This information is largely qualitative and does not allow one to predict the amount of amino acids required under other conditions. Recently, Griffiths and Pirt (*2*) have determined the growth yields of the mouse LS cell from all the essential amino acids and these indicate that in commonly used media, *e.g.*, those of Eagle (*3*) and Waymouth (*4*), growth of the LS cell would be limited by amino acid supply; first glutamine and then leucine and isoleucine would be growth-limiting. This work also shows that the quantitative requirement for amino acids varies during a batch culture. Griffiths and Pirt also found that the most economical utilization of amino acids occurs under steady-state conditions in a chemostat.

ROLE OF SERUM IN CELL NUTRITION

The characterization of the growth-promoting principle in serum is a central problem in the culture of cells. Despite the fair amount of attention it has received, the contribution of serum to the nutrition of cells remains problematical. In general, either cells cannot be cultured without serum or growth is much more reproducible if serum is included in the medium. Serum is an objectionable constituent of media for fairly obvious reasons, not the least because serum must be carefully screened to ensure that it is free from toxic effects. Many workers [Lieberman and Ove (*5*), Michl (*6*), Tozer and Pirt (*7*), and Holmes (*8*)] have shown that the active principle of serum is associated with a specific fraction or fractions in the serum protein. This disproves the early concept that the effects were nonspecific protein effects. The evidence indicates that the so-called α-1-protein in serum is the active fraction, but the isolation of the active fraction in sufficiently large amounts to enable its biological and chemical properties to be characterized has so far proved elusive. It is tempting to suppose that there is some affinity between the serum factor and the so-called nerve growth factor recently isolated by Varon, Namura, and Shooter (*9*).

CULTURE SYSTEMS FOR SUSPENDED CELLS

Batch culture requires the simplest apparatus but almost inevitably it is complicated by the metabolic changes associated with the cycle of lag, growth, and stationary and decay phases. This is illustrated by the fact that rates of metabolism of amino acids are greatest at the beginning of a batch culture and then decrease markedly all through the growth phase. This shows that balanced growth does not occur in a batch culture—if it did, then metabolic rates per cell would settle down to steady values. One can hardly expect balanced growth to occur in a batch culture because of the ever-changing environment to which the cells, presumably are trying to adapt. The transient states of the cells in batch culture could have a considerable effect on the competence of the cells for virus production.

The only way to avoid transient metabolic states during growth is to use the chemostat culture method. A further advantage of this method is that it enables metabolic rates to be varied at will. The greatly extended control over the cell's metabolism in the chemostat seems likely to reveal unexpected metabolic regulations and should show definitively how growth conditions affect cell functions, including virus production. The chemostat is a means of providing cells in a reproducible, well-defined state. It may, however, be difficult to select the optimum state for a particular purpose, such as virus production from the great range of cell states possible in the chemostat.

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Karyologic Characteristics of The WI-38 Diploid Cell System—General Comments [1]

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Vaccines for human use, based on the diploid cell culture system (WI–38), have been studied at this laboratory during the last 3 years. More than half a million doses of such vaccines have already been administered. Control of the cell substrate has been based on the minimum requirements proposed in the Opatija Symposium of 1963 (*1*).

Fifty-five lots of tissue were made and completely tested according to these criteria, and except for a few that had been contaminated in the laboratory, all were suitable for virus propagation.

None of the Opatija standards proved difficult to meet except those imposing an absolute limitation on chromosome abnormalities. The original standards that recommended the rejection of a tissue lot containing any major abnormalities were later amended (*2*) to allow for the actual number of such aberrations occurring in normal cultivation.

To illustrate the range of karyological events that might be expected in large-scale use of the WI–38 cell system, this paper presents a summary of our entire experience. The cytological analyses of 129 WI–38 cultures included microscopic study of about 13,000 metaphases of WI–38 cells.

MATERIALS AND METHODS

Tissue culture.—Production lots (and most experimental cultures) were derived from single ampuls of WI–38 cells frozen at passage 16. The cultures were grown as monolayers in Eagle's basal medium as suggested in the Opatija proposals. Cultures were subdivided on a 2 : 1 or 4 : 1 basis as soon as they were fully grown and were eventually analyzed at passage

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[1] Research supported in part by Public Health Service contract PH 43–63–80 from the Vaccine Development Branch, National Institute of Allergy and Infectious Diseases.

[2] We are grateful to Mr. Albert C. Lowenstein for his statistical assistance.

28 or 29. Several sequential studies were done in which chromosomes were analyzed on alternately successive passages (usually between 20 and 40) of the same ampul-derived culture. For chromosomal analysis, samples of cultures were taken as required and further passed in the same medium with antibiotics and in the presence of 10% fetal calf serum.

Chromosome preparations and karyologic studies.—Chromosome preparations were made on the 2d day after subcultivation, according to the method of Saksela and Moorhead (*3*). Each of the 129 studies consisted of three parts: exact chromosome counts, the search for abnormalities, and an estimation of polyploidy (97 cases). Exact chromosome counts were made at 800× on 100 metaphase cells previously selected for good technical quality under 125×. During the counting procedure each chromosome in each of the 100 cells was examined for chromatid breaks and major chromosome abnormalities. All chromatid breaks (consisting of open disalignment of one or both chromatids, as well as achromatic gaps) were photographed *in situ*. Metaphases with major chromosome abnormalities were subjected to complete photographic reconstruction of karyotype. Finally, 400 metaphases were examined at 320× for polyploidy.

In most cases only one such karyologic study was performed on each ampul-derived culture, usually at passage 28 or 29. Several studies were performed on alternate passages of the same culture to determine whether the abnormalities were persistent and whether in subsequent passages the same or different chromosomes were involved.

The major abnormalities found were of nine general types: dicentric chromosomes, translocations, inversions, chromatid bridges, quadriradial configurations, chromatin duplications, ring chromosomes, acentric fragments, and minute chromosomes. Except for the last two types, all the abnormalities could be assigned to appropriate groups based on the Denver Classification (*4*).

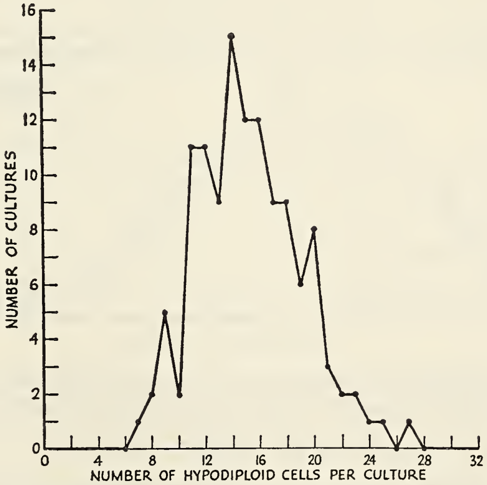
RESULTS

Seven WI–38 cultures (table 1) were excluded from the statistical tabulations because they showed unusual karyological changes or poor growth characteristics that were qualitatively different from the usual range of findings. One of these, #55, maintained a high *p*H (about 7.8) throughout the culture period, in contrast to the usual *p*H drop to about 7.0 or below by day 2. In 2 other cultures, #48a, and 48b, several metaphases showed the same abnormality—a translocation between chromosome 2 and one of the 4–5 group. This unique event, a replicating abnormality, had no parallel among the remaining cultures. In 4 other cultures there were one or more cells with bizarre abnormalities—complex interactions and exchanges involving as many as 5 chromosomes per cell, some defying analysis by karyotype. These were atypical abnormalities, in sharp contrast to the routinely encountered 2-chromosomes-per-cell exchanges (dicentrics, bridges, etc). On cytological grounds each of these 7 cultures seemed sufficiently unusual to warrant separate consideration.

TABLE 1.—Characteristics of 7 cultures excluded from statistical analyses but included in total tabulation shown in table 2

Culture designation	Passage	Percent cells with major chromosome abnormalities	Percent cells with chromatid breaks	Percent polyploidy	Reason for exclusion
48a	28	4	4	0	3 cells of 100 showed same translocation
48b	29	8	2	0	6 cells of 100 showed same translocation
55	29	4	4	2. 5	Poor growth maintenance, elevated *p*H
80	29	5	9	1. 25	Bizarre abnormalities
84a	29	3	10	2. 0	Bizarre abnormalities
84b	29	2	3	1. 0	Bizarre abnormalities
86	29	2	7	2. 0	Bizarre abnormalities

The frequency distribution of hypodiploidy among the other 122 cultures is shown in text-figure 1. No figure is presented for the hyperdiploid metaphases, since the number was very small; approximately 84 cells of 12,200 studied had 47 chromosomes. The excess of hypodiploid counts seems to be a technical artifact caused by the overspreading of chromosomes, since the data fit a normal (random) distribution curve quite accurately.



TEXT-FIGURE 1.—The occurrence of hypodiploidy in 122 cultures of WI–38, including data from passages 13–43.

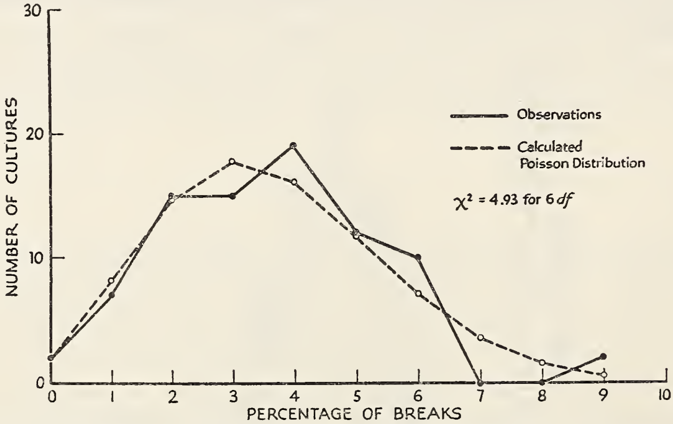
VIRUS VACCINE PRODUCTION

Ninety-seven cultures were examined for incidence of polyploidy. Two of these, at passages 40 and 43, showed an exceptionally high incidence (6.25 and 11.2%, respectively). The remaining 95 cultures, between passages 20 and 34, ranged from 0–4.75%, with a mean of 1.94% and a standard deviation of 0.96%.

All 122 cultures were examined for patterns of chromosone abnormality; of these, 83 were at passages 25–29—the levels suitable for vaccine use. The percentages of cells with either major chromosome abnormalities or chromatid breaks were recorded. To conform with the amended suggestions (*2*), data were stated in terms of the numbers of cells with abnormalities rather than of the numbers of individual chromosomal aberrations. For most cultures, the incidence of chromatid breaks in passages 25–29 was well within the limits set forth in the Opatija proposals. Such breaks occurred as a truncated distribution of random events of low probability that is best described by the Poisson distribution whose limiting value is a "normal" binomial. The theoretical values were obtained from the formula

$$P(x)=\frac{n\cdot\mu^{x}}{x!\ e^{\mu}}$$

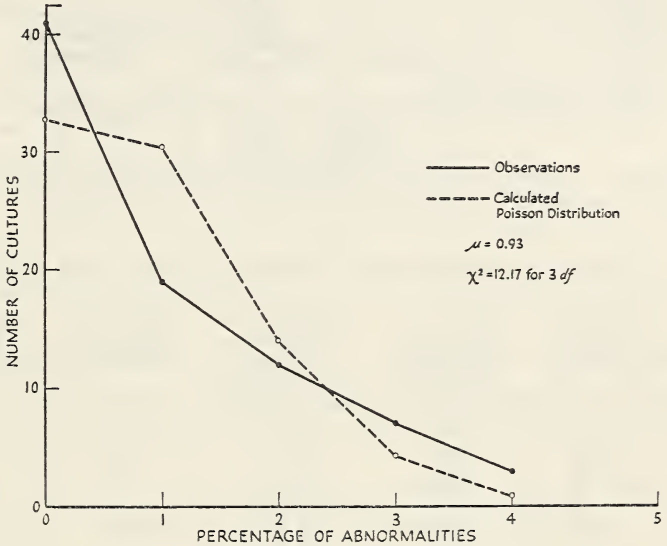
where $P(x)$ is the expected occurrence of x events in n observations, and μ is the mean of all observations. The distribution of chromatid breaks closely follows such a Poisson distribution with $\mu=3.6$, for which the χ^2 is 4.93 with 6 degrees of freedom. Text-figure 2 shows how closely the calculated distribution fits the actual data.



Text-figure 2.—Distribution of chromatid breaks in 82 cultures of WI-38 cells from passages 25–29. Observed data are compared with the calculated Poisson distribution.

The mean incidence of major chromosomal abnormalities (μ) was 0.93. The frequency distribution did not fit a simple Poisson distribution, since it gave a χ^2 of 12.17 for 3 degrees of freedom (text-fig. 3). This failure to fit was caused by a disproportionately high nonoccurrence of abnormalities.

It was possible, however, to calculate the expected number of cultures which *should* have had no visible abnormalities. The most likely number of such zero values was 12. Such a distribution gave an extremely good fit, with a χ^2 value of 0.55. (Thirteen zeros would have given the only slightly higher value of 0.62.) The excess of zero values may be understood by considering the data-gathering problem. Since all abnormalities were photographically verified, the number recorded represents a minimum, because all errors could occur only in the direction of "nondetection."



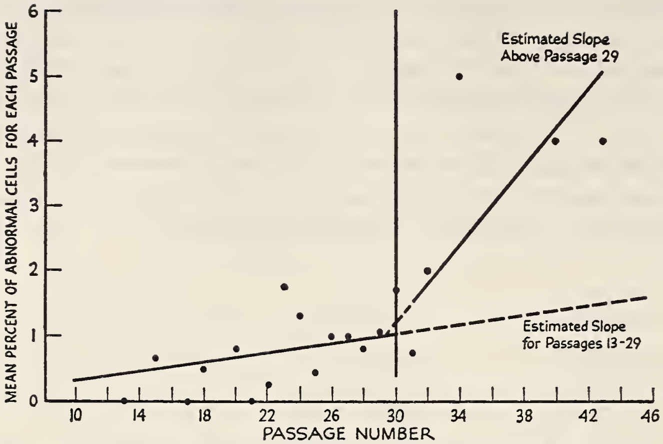
TEXT-FIGURE 3.—Distribution of major chromosomal abnormalities in 82 cultures of WI–38 cells from passages 25–29. Observed data are compared with the calculated Poisson distribution.

There appeared to be a change in karyotype as a function of passage (generation) number. A plot of abnormality against passage (text-fig. 4) indicated *a*) an overall increase in frequency of abnormality with the increase in number of passages and *b*) a marked change in *rate* of increase beyond the 30th passage. The same abrupt change in culture characteristics was also evident in the unusual levels of polyploidy noted in the high passages.

A correlation analysis was not feasible because the number of studies at different passage levels varied considerably. For this reason, the points on text-figure 4 do not have equal weight; the actual numbers of analyses are shown in table 2.

Only one indication of a replicating abnormality was found, in cultures 48a and 48b, which were derived from the same ampul split at passage 22 and passed as parallel cultures.

VIRUS VACCINE PRODUCTION



TEXT-FIGURE 4.—Increase of major chromosomal abnormalities in WI-38 cells as a function of passage level, showing a discontinuity at about passage 30.

TABLE 2.—Passage distribution of cultures in karyologic studies

Passage	Total No. of cultures studied	Average % of cells with chromatid breaks	Average % of cells with major abnormalities	Cultures with polyploidy estimates	Cultures with all vaccine tests completed
13	1	3. 0	0. 0	1	0
15	3	2. 33	0. 67	2	0
17	1	0. 0	0. 0	0	0
18	2	1. 33	0. 50	1	0
20	5	5. 0	0. 80	4	2
21	2	4. 0	0. 0	1	0
22	4	4. 75	0. 50	3	0
23	4	1. 75	1. 75	3	0
24	3	. 33	1. 33	2	0
25	11	3. 73	0. 45	7	3
26	3	2. 33	1. 00	2	1
27	8	3. 75	1. 00	7	2
28	18	3. 53	0. 82	12	7
29	49	3. 62	1. 07	39	34
30	7	5. 0	1. 71	7	5
31	4	6. 0	0. 75	2	1
32	1	4. 0	2. 0	1	0
34	1	4. 0	5. 0	1	0
40	1	2. 0	4. 0	1	0
43	1	6. 0	4. 0	1	0
	129			97	55

DISCUSSION AND CONCLUSIONS

In this detailed study of chromosomes of the WI-38 cell system, the frequency of hypodiploidy accepted by the Opatija Symposium was rarely exceeded, and the limits set for polyploidy were never surpassed within the permitted passage levels.

The incidence of abnormal chromosomes grew as the number of culture passages was increased. The Opatija Conference recognized the occurrence of a relatively high level of simple chromatid breaks, and this level was rarely exceeded in these studies. In the present study, the major abnormalities, *i.e.*, those involving inversions or interactions between chromosomes, also occurred in significant numbers. However, after the examination of a great many replicate cultures and approximately 13,000 metaphases, these events were truly infrequent but randomly distributed and predictable. The failure of the distribution of these abnormalities to fit a normal Poisson curve may be attributable to the nonrandom characteristics of the errors in data collecting; by the nature of the study, underestimation is far more likely than overestimation. After elimination of the cultures showing qualitative differences, the true average frequency of typical abnormalities was about 1%, with a normal distribution in which 4 aberrations would be expected in about 5% of the cultures.

That the distribution of abnormalities (clearly gaussian) is "normal" may also be deduced from the chromatid break data. Since it may be assumed that the "major" abnormalities must be preceded by minor (chromatid) breaks, the distribution of one should reflect the occurrence of the other.

Although the chromatid break data are probably as underestimated as the abnormality data and for some of the same reasons, the underestimation does not greatly affect the characteristics of the distribution. In only very few cultures were no breaks found. The statistical effect of underestimates is seen, therefore, as a downward displacement of the whole curve, with its essentially normal configuration maintained.

In addition to the evidence presented, there are other reasons to believe that random variations within the range reported here have no biological significance. 1) Several recent studies of the chromosomes of normal human cells (*5*) showed that major abnormalities occurred at an average frequency of 1–2% in normal tissues. 2) In published studies of primary (simian) tissues in cultures, higher levels of abnormality were also encountered (*6*). Similar experiences in this laboratory compared with findings in the diploid cell system indicate that normal primary tissues may indeed have a higher frequency of karyologic abnormalities than does WI–38. 3) In the several sequential studies in which a culture of WI–38 was examined at different passages, the abnormalities were random, appearing and disappearing in successive passages and in different chromosomes. These random events were apparently self-eliminating and had no effect on the growth characteristics of cells or cultures, at least within the passage limits (under 30) set by the Opatija recommendations. 4) Finally, the rise in karyologic abnormalities with increased passage, such as was observed in these studies, signals the slowing of growth and the development of "senility" rather than transformation to continuous cell growth.

VIRUS VACCINE PRODUCTION

SUMMARY

WI–38 cells used for vaccine production were evaluated according to the acceptability criteria suggested in the Opatija Symposium of 1963. This experience was consistent with all the minimum recommendations except for the limitations on major chromosomal abnormalities. These occurred at an average rate of 0.93 cells/100 metaphases examined for each culture. Of 129 cultures studied, 7 had unusual chromosomal abnormalities, while the others were distributed in a skewed Poisson distribution. Other cytological characteristics, such as chromatid breaks, hypodiploidy, and polyploidy, occurred as random (normal) distributions and were usually well within the suggested limits. Sequential studies indicated that the frequency of abnormalities rose with passage level—more sharply after the 30th generation.

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GENERAL DISCUSSION

Dr. P. D. Cooper: Dr. Rubin, could you give us a quick rundown on the comparison between production feasibility of the diploid cell line and standard continuous cell line? How many thousand liters could one make from, say, HeLa cells with the same sort of facilities that you were using? Was it much more difficult to use the diploid cell line?

Dr. B. A. Rubin: We have studied the growth in suspended culture, not of HeLa cells but of KB cells of the type you will hear more about from Dr. Green. It is feasible to produce adenovirus in enormously greater quantities in KB cells than in diploid cells. I think that our maximum titers of virus in diploid ran to about 8 logs, whereas we can reach 12 logs of virus in the suspended KB cells without great difficulty. Does that answer the question?

Dr. Cooper: Yes, that's half the question. The other half is: How many cells could you make of the two different sorts with the same facilities?

Dr. Rubin: I'm not sure they are directly comparable. I think there are two factors here. The number of cells produced, I think, would involve approximately the same problem in the same facility. That is, the number of cells would not really differ for the same amount of work and equipment and so forth. However, the productivity of these cells in terms of virus does differ greatly.

Dr. P. F. Kruse, Jr.: I would just like to add a comment to Dr. Hayflick's interesting talk and now to Dr. Rubin's last comment. In considering WI–38 or other serially passaged diploid cell for virus propagation, I think it should be realized that these will form multiple layers with a simple perfusion system, and this might improve virus yields. Whether it would is not known at present.

Just to set the record straight, I would also like to disagree with Dr. Pirt on two counts. One is his statement that physical space is not a limitation in suspension cultures. I feel that it certainly is and frequently plateau phases appear in suspension cultures at relatively low population densities. The other is his statement that you can only get a steady state condition in a chemostat-type suspension culture. Of course, we feel that this too is not so because this can be done easily in a perfusion system for monolayer type cultures.

Dr. S. J. Pirt: How do you substantiate your statements, Dr. Kruse? You're saying that if you have limitation of growth in a batch culture this somehow means that physical space is a factor responsible. How would you rule out the other two possibilities which I mentioned?

With regard to steady states, I do not see how you can get steady states in the cells by anything but chemostat cultures—at least in growing cells. I think it may be possible with nongrowing cells by use of your perfusion technique.

Dr. Kruse: Perhaps we had better define "steady state." I don't mean steady state in terms of cell numbers. I mean it in terms of constancy of the milieu interieur or in terms of homeostasis, in terms of the constancy of the nutrient, *i.e.*, concentrations, *p*H, and so forth. Does that help any or not?

Dr. Pirt: No. What you have got to show is that there is an absolutely steady metabolic rate in the cells.

Dr. Kruse: We have shown this in several ways—one, in a paper published 2 years ago in *Proceedings of the Society for Experimental Biology and Medicine,* in which we reported that the rate of glucose utilization is constant in any particular phase of the proliferation cycle and proportional to the rate of proliferation. We have shown it in terms of the constancy of rate of utilization of essential amino acids. These are some of the ways.

Dr. Pirt: When you say "constancy of utilization" of a nutrient, such as glucose or amino acids, could you take that a bit further? Do you mean you simply observe a constant rate of uptake of these nutrients?

Dr. Kruse: Yes, "uptake" is a better word than "utilization," I suppose. And this is on a per milligram of cell protein basis, not on a per cell basis. Because the size of the cells changes as they become very densely populated, so you have to base this amino acid uptake on a per milligram of protein basis.

Dr. Pirt: Were the cells you were studying actually growing or were they in a stationary phase? The parameters you mentioned undoubtedly are the ones which you have to use and which if they were constant do in fact show that you have a steady state in the cell.

Dr. Kruse: In the experiment to which I am referring, there was a certain rate of proliferation in the multiple-layered cultures, although very much less than in the preconfluent populations.

Dr. Pirt: I think our results certainly showed by such means it is not possible if you do have growth, and this in fact I think is consistent with what other people have found. You notice particularly that in the lag phase there is an enormously greater rate of utilization of certain amino acids—particularly basic amino acids we find—and of glucose. Dr. Merchant's work has shown this with glucose.

Dr. F. Rapp: I would like to ask Dr. Hayflick to make two more comments on his very nice presentation. The first is: Does he have any idea why the human diploid cells are apparently so germfree? Does he feel that enough sources have been adequately tested? Finally, what would he recommend for testing these cells? He mentioned they are not oncogenic but I don't know how this has been done. But what would he recommend for safety-testing these cells for this property, since obviously one cannot use the homologous species?

Dr. L. Hayflick: I don't want to make a categorical statement that these cells are "germfree," as you put it, other than after they are autoclaved. I think that we are concerned here with relative risks. If one is considering the relative risks of using primary cells versus WI–38, it seems quite obvious from all of the work done with WI–38 (they have been tested for viruses repeatedly by several dozen laboratories) that these cells do not appear to have latent viruses by any known test.

As to your second question relevant to oncogenicity, other than satisfying ourselves that they are not oncogenic when inoculated in the hamster cheek pouch, which at best is probably a suspect method, these cells have been inoculated directly into man. Living WI–38 cells at various concentrations had been inoculated into the forearms of several dozen terminal cancer patients some years ago with comparable controls, with the use of HeLa cells on the other forearm.

In the former circumstances WI–38 showed no growth whatever. In the latter situation the HeLa cells proliferated and the nodules had to be excised.

Again this question is one that cannot be completely and absolutely settled, but, as far as the relative merits of cell culture systems are concerned, it is clear to me that certainly not many of you listening here today would want to risk inoculating primary monkey kidney or primary chick cells parenterally into man.

Session II

Characteristics of Cell Culture Systems

Chairman: M. M. Sigel
Rapporteur: Herdis von Magnus

Viral Flora of Chick and Duck Tissue Sources[1]

Roy E. Luginbuhl, *Department of Animal Diseases, University of Connecticut, College of Agriculture, Storrs, Connecticut 06268*

As late as 1946 it was thought that the chicken embryo provided a virus-free growth medium. Beveridge and Burnet (*1*) stated: "There is no well authenticated report of a chick embryo being a natural carrier of any virus, and the technical problem of preventing entry of unwanted viruses to it is immensely simpler than with any type of free-living animal."

Cottral (*2*), in a review on endogenous viruses in the chicken egg, gave 9 diseases of chickens, namely, avian lymphomatosis, avian encephalomyelitis, Newcastle disease, infectious sinusitis, psittacosis, pullorum disease, avian tuberculosis, fowl typhoid, and paratyphoid infections, in which evidence indicated that egg transmission occasionally occurs. Avian adenoviruses (*3*, *4*) have been added to this list, and evidence supporting Cottral's statement has increased (*5*, *6*).

Latent viruses in chicken embryo tissues have also been reported (*3*, *5*, *7*).

I have chosen to discuss all the known viruses of the chicken and duck and will cite evidence for a viral flora in tissues from these species.

CHICKEN VIRUSES

Adenoviruses.—These viruses have been mistaken for lymphomatosis virus (*8–10*) and avian encephalomyelitis virus (*11*). Also described as enteroviruses (*12–18*) and chicken-embryo lethal orphan (CELO) viruses (*4*, *19*), they finally have been classified as adenoviruses (*20–23*). They grow readily in chicken embryos and chicken cell cultures (*8*, *11*, *13*, *14*, *24*) and have been experimentally demonstrated as possible tissue culture contaminants (*25*) and as latent agents in uninoculated chicken-kidney cell cultures (*3*). They have been shown to interfere with propagation of Newcastle disease and influenza A viruses (*26*). These agents are undoubtedly among the most important to consider as latent viruses in chicken tissue sources.

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[1] Scientific Contribution No. 336, Agricultural Experiment Station, University of Connecticut, Storrs, Conn.

Avian encephalomyelitis.—Avian encephalomyelitis is embryo-transmitted and therefore a possible contributor of viral contamination in chicken tissue sources (*27*, *28*). The virus multiplies in chicken embryos (*27*, *29*, *30*) and has been recently described in cultures of chicken-embryo neuroglial cells without a cytopathic effect (*31*). The disease appears to be self-limiting in adult birds and usually does not produce dramatic disease conditions (*6*, *27*, *28*). It has occurred in laying birds without being noticed by the caretaker and therefore could present a problem from the standpoint of contamination of chicken tissue sources.

Avian reoviruses.—Three avian reoviruses were recently described and shown to be related to human reoviruses I, II, and III (*32*). No other work has been reported.

Arthritis.—A virus agent capable of producing arthritis in chickens has been described (*33–36*). It has been propagated primarily in chicken embryos (*33*, *34*, *36*) but also has been identified growing in chicken-embryo fibroblast cultures with a cytopathic effect (*35*). Nothing has been reported concerning the pathogenesis. With chicken embryos, the agent resisted 60°C for 8 hours, 20% ether, 0.5% chloroform, and produced cytoplasmic inclusions in the chorioallantoic membrane.

Fahey-Crawley virus.—This virus was first described in conjunction with chronic respiratory disease, which is caused by *Mycoplasma gallisepticum* (*37*). It has been found to cause a nasal discharge in chickens under 3 weeks of age but there were no signs of disease in older birds (*38*). It has been described as a poor antigen, which is stable at 56°C for 90 minutes and stable at *p*H 2.0 and 12.0 (*39*). No information was found concerning propagation in cell cultures or embryonic transmission.

Fowl plague.—This disease is not found in poultry flocks in the United States and therefore should not be a problem in chicken tissues obtained in this country. The virus has, however, been propagated in cell cultures (*40*) and has been studied with the use of fluorescent antibodies (*41*).

Fowl pox.—This virus does not appear to be embryo-transmitted. The viremia reportedly disappears as soon as visible lesions disappear (*42*). It does not appear to be a likely contaminant of chicken tissues.

Infectious bronchitis.—This virus infection is very common in poultry and is self-limiting in nature. It usually persists for about 3 weeks and then immunity is present. Vertical transmission is not a problem in the spread of this disease, even though the virus has been demonstrated in eggs for 43 days and in tracheal exudates for 28 days after infection (*43*). Most strains produce obvious respiratory signs and a drop in egg production. Poultrymen usually vaccinate their birds during the growing period to prevent the disease from occurring during the laying period. Different serologic strains have been described (*44*, *45*). The virus grows readily in chicken embryos (*46*, *47*), chicks (*48*), and cell cultures (*49–52*). Though not described as a contaminant of cell cultures, it certainly has the necessary characteristics to become a candidate for this distinction.

Infectious bursal agent.—This disease was first described by Cosgrove (*53*) in 1962 and is highly contagious. Contaminated environment has been shown to remain infective for 122 days. The specific mode of transmission appears to be related to contaminated feed or water. The agent has been described as being 10–50 mμ in diameter, stable at 56°C for 5 hours, labile at pH 2.0, and stable in ether and chloroform (*54*). Chicks can be infected intraocularly; necrosis of lymphoid elements occurs 3–4 days post inoculation (*55*). Propagation in chicken embryos from certain flocks has been reported, but no record of cell culture propagation is available.

Infectious laryngotracheitis.—This avian herpesvirus (*56*) grows well in embryos (*57–59*) and in cell cultures (*59–63*). The virus has not been described as being transmitted vertically and therefore is not likely to be involved in vaccines prepared from chicken embryos or chicken-embryo cell cultures. An excellent review of the literature on infectious laryngotracheitis is available (*64*).

Lymphoid leukosis.—Research on viruses responsible for lymphoid leukosis has resulted in increased knowledge concerning this virus-induced neoplastic disease of chickens. That the virus lives and multiplies in chicken embryos and cell cultures without causing a pathogenic effect (*5*) has led to a whole series of new findings concerning the nature of this cancer-inducing virus. The pattern of congenital transmission has been determined (*65*, *66*) as well as the demonstration of an immune tolerance phenomenon (*67*). Development of immunity after contact infection indicates that this mechanism also plays a role in this disease (*67*). The concept of virus defectiveness is also an outgrowth of research on lymphoid leukosis (*68*).

The fact that lymphoid leukosis viruses can produce protein coats for Rous sarcoma virus particles and that Rous sarcoma virus is capable of producing tumors in a wide variety of species has caused great concern among regulatory officials (*69–72*). Methods have been developed that can be used for detection of lymphoid leukosis antigens and antibodies (*73–79*), and also for the development of flocks free of this infection (*80–82*).

Embryo transmission is well established (*5*, *65–67*, *83–85*). Contamination of biologic products produced from chicken tissue sources is also a fact (*86*,*87*).

Studies on this virus indicate that more than one serologic group exists and should be accounted for in all surveillance of biologics (*88*).

Marek's disease.—Our understanding of the avian leukosis complex was considerably advanced when lymphoid leukosis was considered as a separate disease entity from Marek's disease (*89–92*) and when Marek's disease was transmitted repeatedly in susceptible chicks (*93*). The agent is extremely cell-associated and has been transmitted primarily with tumor cell suspension or whole blood (*94*). There is little evidence published to date to support embryo transmission. However, the field incidence of this disease is extremely high and is estimated to be costing the poultry industry at least $150 million annually.

The recent announcement of a herpes-like virus being associated with the disease is most intriguing and opens a new area for investigation (*95*).

Myxovirus.—This virus was isolated from chickens with a mild respiratory disease (*96*), and since the first isolation, 15 new agents from chickens, turkeys, and ducks have been reported (*97*). These agents have not been studied extensively.

Newcastle disease.—There is evidence that this respiratory disease virus can persist in a flock for 2–3 months after recovery (*98*) and in eggs produced by such a flock for a similar period (*99*, *100*). The virus has also been isolated from eggs of hens vaccinated with live Newcastle disease vaccine (*101*), and from chicks, embryos, and infertile eggs during a natural disease (*102*, *103*). There is, therefore, good evidence of this virus being a possible contaminant of tissues from chicken sources, depending on the disease status of the flock. Newcastle disease virus has been isolated from poultry biologics produced for other diseases (*104*) and has been described as a contaminant in uninoculated chicken-embryo fibroblast cultures (*7*). In these cultures a cytopathic change occurred in cells 2–4 days after the growth medium was changed. The virus was considered to be present in the latent form.

T virus.—This virus has been considered as a lymphomatous agent (*105*); however, it is not related to lymphoid leukosis viruses or to the Marek's disease agent as determined by currently available methods. It was isolated from turkeys and has been an experimentally induced disease of chickens.

DUCK VIRUSES

Relatively little research has been reported on the search for latent viruses in tissues obtained from duck sources.

Duck plague.—This disease has been only recently described in the United States (*106*, *107*). The virus was readily propagated in chicken- and duck-embryo cell cultures, producing a cytopathic effect and plaques after several passages. Transmission occurred readily by intramuscular, oral, and intranasal inoculation and by cloacal swabbing. Inclusion bodies were observed in affected cells and were determined to be made up of DNA. Inactivation occurred in 10 minutes at 36°C and at pH 3.0 and 11.0 Measurements of the outer diameter were determined to be 180 and 75 mμ for the inner core. Classification was most likely in the herpesvirus group (*106*).

Eastern encephalitis.—This arbovirus was isolated from ducklings obtained from 5 farms on Long Island (*108*) and later was propagated in duck-embryo fibroblast cultures, producing a cytopathic effect and plaques

(*109*, *110*). Both A and B groups of arthropod-borne viruses have been propagated in duck-kidney cell cultures (*111*).

Hepatitis.—This disease, first described in 1950 by Levine and Fabricant (*112*), has been reported to persist throughout the hatching season after infection under natural conditions (*113*). Virus has been demonstrated in the brain, lung, heart, liver, spleen, and leg muscles of inoculated ducklings. Inoculated chicken embryos contain virus in the allantoic-amniotic fluids, head, liver, and chorioallantoic membrane (*114*). Duck-embryo kidney cell cultures support the growth of the virus (*115*), whereas duck-embryo liver cells (*116*) and duck embryo fibroblasts (*117*) do not appear suitable for continuous virus propagation. Chicken-embryo liver cells will support virus growth without a cytopathic effect (*118*) as will chicken embryo cells prepared by the use of collagenase instead of trypsin (*119*).

In summary, it appears that the avian adenoviruses and lymphoid leukosis viruses are excellent candidates for viral contaminants of chicken tissue sources, whereas avian encephalomyelitis, infectious bronchitis, and Newcastle disease may be candidates provided the flock is in the active stages of infection. Infectious laryngotracheitis virus is not likely to be found in avian tissue sources. There has not been sufficient research on the other viruses mentioned to classify them according to the chances of finding them in chicken tissue sources.

Duck hepatitis could be considered as a possible candidate; however, insufficient research has been reported to draw conclusions on duck plague. Eastern encephalitis is not likely to be found in duck tissues.

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Viral Flora of Chick and Duck Tissue Sources—Discussion

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MOST chicken and duck viruses are potential contaminants of cultures prepared from adult tissue. More important, however, is the smaller number of viruses naturally transmitted via the embryo, since the cultures most often used for research or vaccine production are derived from this source. Dr. Luginbuhl has discussed the recognized avian viruses and has incriminated adenoviruses and lymphoid leukosis viruses as the most likely contaminants of chick cell cultures. Both viruses are ubiquitous in commercial flocks, rarely cause clinical disease and are embryo transmitted. The presence of these viruses cannot be reliably detected by alterations in culture morphology. Adenoviruses often exhibit latency (*1*) and lymphoid leukosis viruses are considered noncytopathic although transformation of chick-embryo fibroblast cultures may occur after extended culture periods (*2*).

Also extremely prevalent in commercial chickens is Marek's disease, a highly contagious disease characterized by proliferation and neoplasia of lymphoid tissue. A cell-associated herpesvirus recently isolated from Marek's disease has been circumstantially incriminated as the etiologic agent (*3*, *4*). The virus grows in both chick and duck cell cultures; however, disease manifestations in ducks have not been reported. Epidemiological observations have suggested that the causative agent may occasionally be transmitted via the embryo, although laboratory confirmation of this has not been reported. The virus should be considered as a potential contaminant of embryo cell cultures and a likely contaminant of cultures prepared from adult tissue. However, due to the close association of the virus with intact cells, its presence in processed vaccines seems unlikely.

All embryo-transmitted viruses may be considered justifiably as potential contaminants of embryo cell cultures. However, since virus shedding may be sporadic or transient, substantial evidence is required to negate the possibility of embryo transmission. Many of the viruses mentioned in the previous paper, *e.g.*, reovirus, arthritis agent, Fahey-Crawley virus, infectious bursal agent, influenza-A-related myxoviruses and the several duck viruses, have not been studied sufficiently to eliminate the possibility of embryo transmission. Successful maintenance of chicken and duck populations free of potential cell culture contaminants will require the use of a list of all possible candidate viruses plus the development and intelligent application of assay techniques for each.

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[1] U.S. Department of Agriculture.

In summary, the role of adenoviruses and lymphoid leukosis viruses as contaminants of chick-embryo cell cultures appears well established. Representatives or relatives of both virus groups have proved to be oncogenic for mammals (*5*, *6*) and both should survive in vaccines prepared from infected cultures. Other chicken viruses such as avian encephalomyelitis, infectious bronchitis, and Newcastle disease are also transmitted via the embryo but are less frequently encountered. Little or no information on the viral flora of duck embryo cells is available.

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GENERAL DISCUSSION

Dr. J. L. Melnick: I would like to ask the previous two speakers a question about Marek's disease. In view of the fact that the herpes-type virus, which has been isolated from the cells of animals having this disease, has not yet produced the disease in any inoculated animal, what is the evidence by which this virus has been associated etiologically with the disease? In answering the question, would you also comment on whether those cells which produce the disease always contain the herpesvirus or whether cells free of virus particles also can transmit disease.

Dr. R. L. Witter: The experimental evidence for the implication of the herpesvirus in the etiology of Marek's disease is entirely circumstantial at the present time. We have not yet been able to achieve an agent free of cells with which to work. Under a number of conditions and in two entirely separate laboratories, a very similar virus has been observed. Present in inoculated cell cultures, it produces a cytopathic effect in these cultures. The correlation between cytopathogenicity, bird infectivity, and herpesvirus particles as revealed by the electron microscope has been very high to date. This type of evidence leads us to think that this virus and Marek's disease may be causally related. Obviously, a considerable amount of work remains to be done before this can be determined with any certainty.

Dr. Melnick: Do cells that are able to transmit the disease always contain virus particles?

Dr. Witter: The virus has been observed only in inoculated tissue culture cells. It has not been observed in cells obtained directly from the chicken. Of course, these latter cells are able to transmit the disease.

Also a very significant property of the disease is this ability to transmit by direct contact, apparently through particles borne through the air, and to survive in the manure for extended periods under fairly dry conditions, conditions that would essentially preclude the survival of intact cells. Evidence of this sort indicates that there may be a cell-free virus or an etiologic agent present, and this constitutes a discrepancy in the picture of the disease that we have not yet resolved.

Dr. M. M. Sigel: Has this virus been characterized serologically? If so, is there any association of antibody levels with infection?

Dr. Witter: This virus has not been characterized serologically. At the time, there has been no evidence of a natural antibody to the disease, although we hope to find such an antibody as soon as tools are available with which to work.

Viruses Infecting Cattle and Their Role as Endogenous Contaminants of Cell Cultures

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For the purposes of this Conference, the viruses known to infect cattle will be reviewed principally with reference to their role as endogenous contaminants of cell cultures. Further, since even a brief consideration of all the well-recognized virus types would be much too long, the discussion will be limited to viruses representative of their group. The classification scheme followed here is that adopted, for the present, by the Western and Eastern Hemisphere Committees on Animal Virus Characterization (*1*).

DNA-TYPE VIRUSES

Representative viruses of the DNA type, known to infect cattle, are presented in table 1.

Adenoviruses

Although the original isolations of the three recognized types of bovine adenovirus were made from healthy animals (*2–4*), subsequent studies have shown that at least types 1 and 3 are pathogenic for calves (*5–7*). The participation of these viruses in respiratory disease outbreaks of cattle was revealed by broad serological surveys conducted in Britain (*8*), and additional serological and virus isolation data (*6*, *9–11*) demonstrated the high incidence of adenovirus infection in cattle populations.

Adenovirus infection, particularly in calves, may lead to a catarrhal inflammation of the upper respiratory tract as well as to pneumonia and enteritis. The degree of involvement may be mild or acute. In asymptomatic animals, virus has been recovered from conjunctiva and respiratory and intestinal tracts. In calves showing flank disease, virus has been recovered from conjunctiva, nasal and tracheal mucosa, lungs, intestinal tract, lymph nodes, spleen, and kidney, but apparently not from blood (*7*, *10*). In

Table 1.—DNA-type viruses infecting cattle

Virus group	Proposed designation	Common designation
Adenovirus	Bovine adenovirus 1 Bovine adenovirus 2 Bovine adenovirus 3	Bovine #10 Bovine #19 WBR-1
Herpesvirus	Bovine herpesvirus 1 Bovine herpesvirus 2 Bovine herpesvirus 3 Porcine herpesvirus	Infectious bovine rhinotracheitis Bovine mammilitis Allerton Pseudorabies
Unclassified		Malignant catarrh
Papovavirus		Bovine papilloma
Poxvirus		Vaccinia Cowpox Bovine papular stomatitis Lumpy skin disease (Neethling) Pseudocowpox

experimental trials, virus was recovered from tracheal exudate, intestinal mucosa, and feces 70 days after exposure (*10*). There is no evidence of fetal infection with the agents.

Neutralization of bovine adenoviruses types 1 and 2 with human γ-globulin has been reported (*11*), but there is no evidence that bovine adenoviruses are infective to humans.

Type-3 virus has been shown to induce formation of tumors in hamsters (*12*) and of specific tumor and T antigens (*13*).

Herpesviruses

Bovine herpesvirus 1.—The virus is the etiologic agent of infectious bovine rhinotracheitis (*14*, *15*) and also causes conjunctivitis (*16*), infectious pustular vulvovaginitis (*17*, *18*), encephalitis (*19*), and abortion (*20*). Infections with the virus are known to occur in all of the United States, Canada, Britain, most European countries, the Near East, North Africa, New Zealand, and Australia. The incidence of infection with the virus is high, particularly in dairy and feedlot herds where respiratory disease predominates. Although morbidity may be high, occasionally involving 100% of the population, mortality rarely exceeds 5% (except in encephalitis, where mortality is over 50%) (*21*).

The virus is highly cytopathogenic in bovine cell cultures and can be isolated readily during the acute phase of respiratory infection from nasal and tracheal exudates and conjunctiva, and less frequently from lungs. In the bloodstream the virus is concentrated in leukocytes and can be isolated from the buffy coat (*21*). In genital infections isolations can be made from vaginal and preputial exudates. Virus can be isolated from most tissues of the fetus in abortions (provided the fetus is not greatly autolyzed) and from cotyledons (*22*). A study of experimental respiratory infection (*23*) yielded virus isolates from various sites of the upper respiratory tract, lungs, regional and distant lymph nodes, lachrymal ducts, conjunctiva, esophagus, kidney, and brain; in this study the virus did not persist beyond 12 days post infection. On the other hand, epizootiological and virus isolation data indicate that animals may harbor the virus for at least 3 months after infection (*23*, *24*).

Bovine herpesvirus 2 (*bovine ulcerative mammilitis virus*).—The virus causes localized lesions of the teats and skin of the udder. The disease is relatively common in milking herds in Britain. The virus has some antigenic relationship to the Allerton virus, but is not related to herpes simplex (*25*).

Bovine herpesvirus 3 (*Allerton virus*).—The agent is one of the viruses isolated from cases of lumpy skin disease of cattle, which is confined to Africa (*26*, *27*). In experimental animal trials, the virus produced localized necrotic lesions but no systemic involvement (*27*).

Porcine herpes (*pseudorabies*) (*28*).—Pseudorabies in cattle is an extremely acute disease characterized by the development of severe pruritus followed by signs of central nervous system involvement. The incubation period is 3–5 days. Animals usually succumb 36–96 hours after signs appear. Cattle become infected due to contact with swine carrying the virus (*29*); cattle-to-cattle transmission does not occur. The virus is infective to humans (*30*).

Unclassified: Malignant Catarrhal Fever (*31*)

This acute disease of cattle is characterized by inflammation of the mucous membranes of the upper respiratory tract, ocular changes, and involvement of the central nervous system. The disease probably occurs in all parts of the world. Usually only sporadic single cases appear in a herd, but in feedlots the incidence of the disease may be considerably higher. Mortality is over 98%. The mode of transmission is not well defined. The virus is thought to belong to the herpesvirus group (*32*).

Papovavirus

Bovine papilloma virus.—This causes a transmissible disease in cattle characterized by formation of papillomas of the integument. The disease is world-wide in distribution. The warts most commonly occur on the skin of the head and neck, abdomen, back, and udder, but also on genital organs, teat ducts, esophagus, tongue, and rumen (*33*). There is no systemic involvement and the infection is self-limiting.

Although there are reports of experimental virus transmission and papilloma development in humans (*33*), the transmission probably does not occur in nature (*34*).

Poxviruses

Vaccinia.—Transmission of this virus by milkers to cattle rarely, if ever, occurs in the United States at the present time (*35*).

Cowpox.—The virus, which commonly affects the skin of the udder and teats of cattle, is considered to be distinct from vaccinia. Infection leads to formation of papules and vesicles followed by crusting. There is no systemic involvement. The virus is infectious to humans (*35*).

Bovine papular stomatitis.—The virus causes a rather benign disease characterized by the development of papules, followed by ulceration, on the muzzle and in the mouth of young calves. The infection usually runs a short course and there is no systemic involvement. The disease is known to occur in Europe, Africa, and North America. Unfortunately the condition has been alluded to by various descriptive terms, such as ulcerative stomatitis, pseudoaphthous stomatitis, and erosive stomatitis, with resultant confusion as to its etiology.

The virus isolated so far studied in different parts of the world appears structurally identical and definitely belong to the pox group. Morphologically they seem identical with the Orf virus of sheep. However, additional comparative studies are necessary to establish whether the strains are homologous.

Lumpy skin disease.—The Neethling virus is considered the etiologic agent of the generally more severe form of lumpy skin disease (*36*, *37*). The disease occurs in the cattle of South and East Africa. It is characterized by the formation of multiple nodules in the skin, lesions in mucous membranes and viscera, and by adenitis.

Pseudocowpox.—This virus is similar to cowpox. The virus, however, is distinct from the latter. It is infectious to humans, causing a lesion commonly known as milker's nodule.

RNA-TYPE VIRUSES

Representative viruses of the RNA type are presented in table 2.

Myxoviruses

Rinderpest (*38*).—This disease of cattle is now confined to parts of the Near East, Asia, and Africa. Infection of susceptible cattle with a virulent strain of the virus results in an extremely acute disease, characterized by inflammation and ulceration of mucous membrances of the alimentary tract and necrosis of lymphoid tissues. In such cases morbidity is 100% and mortality may be above 90%. The virulence of virus strains and the susceptibility of different breeds of cattle, however, are variable, and infection may be so mild as to be unrecognized. Transmission is through contact with infected animals, which shed virus during the incubation period. During the acute phase, virus is present in the urine, feces, and exudates. Viremia is present.

TABLE 2.—RNA-type viruses infecting cattle

Virus group	Proposed designation	Common designation
Myxovirus		Rinderpest
	Parainfluenza 3	SF–4
Picornavirus		Foot-and-mouth disease
	Enterovirus	Numerous isolates
	Rhinovirus	SD–1
Reovirus	Reovirus types 1, 2, 3	SD–1
Unclassified		Bovine virus diarrhea
M.R.H.E.V.*		Rabies Vesicular stomatitis

*Miscellaneous RNA-helical-envelope viruses.

Parainfluenza 3 (SF–4).—The virus is associated with a common respiratory syndrome of cattle termed "shipping fever" (*39*) and was originally isolated from lung tissue and nasal mucus of cattle affected by the disease (*40*). Experimental exposure of calves to aerosolized virus resulted in a mild upper respiratory infection (*40*). Since the original isolation, the virus has been frequently recovered from both diseased and well animals. Serological studies have revealed that infection of cattle with this virus is high. In one survey (*41*) 68% of the calves from several herds were found to have significant antibody levels to the virus.

The bovine parainfluenza 3 virus has been incriminated as a cause of bovine abortion because it was isolated from the thoracic fluid and lung tissue of an aborted fetus (*42*). *In utero* inoculation of 4 fetuses with the virus (admittedly a drastic experimental approach) resulted in a generalized infection of 3 (demonstrated on days 4–7 post inoculation). The fourth fetus aborted on day 9 post inoculation, but the virus was not recovered (*43*). Strains of bovine parainfluenza 3 virus have been serologically differentiated from the human type strains (*44*). There is no evidence that human strains can naturally infect cattle, or that cattle strains can infect humans (*45*).

Picornaviruses

Foot-and-mouth disease (46).—The vesicular disease which is caused by the viruses comprising this group is still economically the most costly infection of cattle. The disease occurs in most of the cattle-raising regions of the world, and cattle only in Australia, New Zealand, and Central and North America have remained free of infection for extended periods.

Infection of animals with the viruses leads to the formation of vesicles, in certain mucous membranes and portions of the skin, and necrosis accompanied by a general systemic reaction. The feature of the infectious process of particular significance for the present discussion is that within 24–48 hours after the multiplication of the virus begins in the epithelium, the virus is disseminated through the blood to all the tissues of the animal. Generally, the tissues become free of infective virus after a few days, but there is also evidence that occasional animals may remain carriers for long periods. The virulence of strains is variable and inapparent infections may occur. Since morbidity is high and mortality generally quite low, the problem of carrier animals assumes significance. The virus is infective to humans and is mildly pathogenic.

Enteroviruses (*47*).—The isolation of bovine enteroviruses in the past 10 years by numerous research workers of several countries has been, to say the least, stupendous. Although isolations have been made from well and diseased animals, the general opinion is that most viruses are not associated with disease and do not produce generalized infections. However, repeated isolation of enteroviruses from fetuses and uterine tracts of cattle in Britain (*48*) strongly suggests the participation of these viruses in infertility problems.

Bovine rhinovirus (*strain SD-1*).—The virus was isolated from nasal secretions of a calf with acute rhinitis. Transmission studies in colostrum-deprived calves have shown that the agent has an affinity for the upper respiratory tract (*49*).

Reoviruses.—Types 1, 2, and 3 have been isolated from cattle. The strains are indistinguishable from human isolates (*50*). Longitudinal studies in cattle herds did not associate these viruses with any specific disease, nor did experimental exposure of calves with the viruses produce any illness (*51*). Infectivity of human strains to cattle, and of cattle strains to humans, has been demonstrated.

Although bovine reoviruses have not been associated with any specific disease, this does not preclude the possibility that the agents may disseminate and invade cells of various tissues. Examples of this are well documented in other animal species (*52*, *53*).

Miscellaneous RNA Helical Envelope Viruses

Bovine virus diarrhea.—The acute form of the disease caused by the viruses of this group is characterized by fever, nasal discharge, erosions of the mucosa of the alimentary tract, and diarrhea (*54*, *55*). The disease is known to occur in most of the United States, Canada, England, most of Europe, Australia, and New Zealand. The incidence of the infection in the United States, as evaluated by serological surveys, is between 50 and 60% (*56–58*).

Although acute outbreaks of the disease occur, accompanied by high mortality, most of the infections are subclinical. Isolation of the virus from natural or experimental cases during the febrile stage of the disease can be made from blood, spleen, and lymph nodes. In subclinical infections the virus can be most readily and repeatedly isolated from washed leukocytes, even when serum antibody is present (*59*).

Of particular importance to the present topic of discussion is the now well-recognized and confirmed intrauterine infection of calves. The evidence for such infections is based on the observation that newborn and day-old calves may be carriers of the virus (*60, 61*); bovine cell cultures may be contaminated with the virus through the medium of fetal bovine serum (*58, 62*), and the virus may be present in primary cell cultures derived from fetal bovine tissues (*63*).

Rabies (*64*).—Thousands of cattle die of rabies infection annually, but since the virus does not disseminate in the tissues during the incubation period and the animals die rapidly after the appearance of signs, the virus can be disregarded as a potential cell culture contaminant.

Vesicular stomatitis (*65*).—The virus causes an acute "vesicular disease" in cattle with symptoms very similar to those of foot-and-mouth disease. The disease is enzootic in many areas of this country and in other parts of the world, and outbreaks are not uncommon. The short incubation period, the acuteness of the illness, and the characteristic symptoms probably eliminate the virus as a cell culture contaminant.

DISCUSSION

Potential viral contaminants of cell culture can be agents which:

1) show high incidence of infection in the host; 2) produce a systemic infection and disseminate to tissues commonly used in cell culture, or have a specific tropism for tissues commonly used in cell culture; 3) remain in the tissues of the host for a sufficiently long time to make their isolation likely; 4) produce a silent, or at most a mild disease; and 5) will replicate or at least survive under *in vitro* cell culture conditions.

Among the viruses considered in this review, the following satisfy all or most of these conditions: adenoviruses, bovine herpesvirus 1, parainfluenza 3, at least one enterovirus strain, bovine virus diarrhea virus, and perhaps reoviruses.

Of the above, herpesvirus 1, parainfluenza 3, the enterovirus, and bovine virus diarrhea virus are especially significant, since they may be encountered in both fetal and adult animal tissues. Additionally, the fact that many of the bovine virus diarrhea virus strains are noncytopathogenic makes this virus a particular problem in cell culture contamination.

SUMMARY

A review of the viruses known to infect cattle indicates that the bovine adenoviruses, bovine herpesvirus 1, parainfluenza 3 virus, certain entero-

viruses, and bovine virus diarrhea virus may be considered potential contaminants of cell cultures.

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Viral Flora of Canine Tissues

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In general, the only viruses isolated from the dog produce disease. By 1967 eleven viruses had been isolated from the dog. The dog has no pox-like disease, so it presumably is free of pox viruses.

The classification scheme of the Western Hemisphere Committee on Animal Virus Characterization is used in this paper.

Until recently the only known virus that definitely caused respiratory distress in the dog was its most important viral pathogen—canine distemper virus (*1*). Canine distemper virus is tentatively classified as a myxovirus closely related to measles and rinderpest viruses (*2*). It has an affinity for epithelial tissue; consequently it produces signs of illness referable to many systems in the dog, including the respiratory system. There is growing evidence that a parainfluenza virus, a canine herpesvirus, and a reovirus also are capable of producing respiratory signs of illness in the dog.

Infectious canine hepatitis (ICH) virus, an adenovirus with an affinity for endothelial and hepatitic cells, causes a severe hemorrhagic and edematous disease in puppies (*3*). At present most individuals agree that there is only one immunogenic type of ICH virus. One strain isolated in Canada from tonsillar tissue does show some antigenic and pathological differences from other strains of ICH virus (*4*). There is a group complement-fixing antigen shared by ICH virus and other adenoviruses, but there is no cross-neutralization (*3*).

Rabies virus is well documented (*3*) and known to be an important canine pathogen. It is tentatively placed in a miscellaneous-RNA-helical-envelope virus group (*3*).

Two members of the herpesvirus group produce disease in the dog. Pseudorabies generally is not associated with dog pathogens, but there is evidence it may be important in dogs of the southern United States (*3*). Recently, a canine herpesvirus that produces disease in pregnant bitches with severe consequences to their embryos and infant offspring was isolated and described (*5–8*). In more recent reports on this disease the virus was incriminated as a cause of respiratory distress (*9, 10*) and also encephalitis

(*11*). Thus the dog virus has the same pathogenic potential as the human and bovine herpesviruses. Comparisons of U.S., French, Canadian, and English strains of canine herpesvirus indicate there is one known serotype thus far.

TABLE 1.—Viral flora of the dog

Viral group	Name of virus	Pathogen for dog	Tissue affinity
Adenovirus	Infectious canine hepatitis.	+	Endothelial and hepatic cells.
Enterovirus	ECHO virus 6	+	Enteric.
Herpesvirus	Canine herpes	+	Reproductive tract of pregnant females and fetuses, respiratory tract, nervous tissue.
Herpesvirus	Pseudorabies	+	Nervous tissue, lungs, heart, and skin and its underlying tissues (pruritis).
Miscellaneous RNA-helical-envelope virus.	Rabies	+	Nervous tissue and salivary glands.
Mxyovirus	Canine distemper	+	Epithelial tissue.
Papovavirus	Canine papilloma	+	Oral cavity and lips.
Paramyxovirus	Mumps	+	Parotid gland and saliva.
Reovirus	Canine reovirus I	+	Enteric and respiratory tracts.
Unclassified	Mast cell leukemia	+	Proliferation of mast cells in blood and visceral organs.
Unclassified	Canine veneral tumor.*	+	Reproductive organs of female and male.

*Questionable viral etiology.

Reovirus I has been isolated from dogs with respiratory signs of illness (*12*). Studies by Lou and Wenner with their isolate showed that interstitial pneumonia was produced in naturally and experimentally infected dogs. The inoculation of this same strain in germfree and specific pathogen-free dogs failed to produce signs of illness or pathological changes but it did cause infection (*13*). Massie and Shaw (*14*) recovered reovirus I from 4 of 133 dogs. In 4 experimental puppies, one of their isolates produced signs of illness referable to the respiratory and enteric tracts.

ECHO virus type 6 has been isolated from rectal swabs in 21 of 29 healthy beagles (*15*). Neutralizing antibodies in dilutions up to 1:8 have been found in the sera of a few dogs (*16*). Pindak and Clapper (*17*) produced an enteric disease in 6 experimental puppies that were fed tissue-cultured virus. The incubation period varied in the dogs, but none had diarrhea after 21 days post feeding. The neutralizing titers in all 6 experimental dogs were less than ¼.

A report in the literature states that mumps virus was isolated from the saliva of 2 puppies with swollen parotid glands (*18*). Both cases occurred during outbreaks of mumps in their 2 different households. The viruses isolated from the saliva of the puppies possessed properties similar to mumps virus.

Perhaps the most interesting and productive future studies in canine virology will occur in the field of canine oncology, which is certainly of marked interest to this Conference. Canine papillomatosis is definitely caused by a virus (*3*). Cell-free transmission of a canine mast cell leukemia evidently has been accomplished on two different occasions in different breeds, suggesting that one or more leukemogenic viruses are present as spontaneous infections in natural dog populations (*19*, *20*). A venereal tumor that occurs in the dog is known to be transmitted by coitus; its cause ultimately may be proved to be other than a virus, but there is little doubt of its infectious nature (*3*).

At present we recognize that 11 viruses have been isolated from the dog. In this group, canine distemper and ICH occur as common infections which most dogs have during their first year of life. This may also be the case with parainfluenza and canine herpesvirus, but these agents just recently have been isolated from the dog and there has not been time to develop a large body of information about their incidence in the dog population. The incidence of canine herpesvirus infection is high in dog colonies where the infection exists (*21*). The incidence of rabies and pseudorabies among dogs is low but invariably fatal (*3*). Reports of complement-fixing antibodies in normal dog sera suggest that a low percentage of the dog population has been exposed to mumps virus (*22*). Reovirus I and ECHO virus type isolated from the dog are similar to, if not identical with, the human viral pathogens. The incidence of these infections in the dog has not been completely studied, but present information suggests that 1 of every 3 has been exposed to reovirus I (*12*, *14*). The incidence of canine papilloma and canine mast cell leukemia is unknown (*3*), since there is no reliable serological method of determination; however, the clinical occurrence of these two entities as well as the canine venereal tumor (*23*) is infrequent.

In some canine viral infections, persistence of virus in certain tissues is correlated with signs of illness and/or pathological lesions. This is true of canine distemper, rabies, and pseudorabies. ICH virus presumably persists in the kidney, with little or no microscopic evidence of pathological alterations in carrier animals. There is also evidence that ICH virus persists in the tonsils. It is known to persist in urine at least 9 months (*24*).

VIRUS VACCINE PRODUCTION

Table 2.—Biological properties of canine viruses influencing selection of cell cultures for vaccine production

Name of virus	Incidence in adult dog population	Persistence of Virus		Evidence of oncogenicity	Produces infection in man
		Tissue or excretion	Known period (weeks)		
Infectious canine hepatitis.	High	Urine Kidney Tonsils	39 Unknown Unknown	+	—
Canine distemper.	High	Footpads Brain	4–6 6	—	—
Canine herpesvirus.	High in infected dog colonies.	Turbinates Kidney Nasal	3 Unknown 3	—	Unknown
Reovirus I	Low to moderate.	Spleen	3. 5	—	+
Rabies	Low*	Nervous tissue Salivary gland	16–20 Terminal stage of disease.	—	+
Echo virus type 6.	Low	Feces	5	—	+
Mumps	Low	Parotid gland	Unknown	—	+
Canine parainfluenza.	Unknown	Respiratory tract.	Unknown	—	Unknown
Pseudorabies	Low*	Brain tissue, skin, and underlying tissues.	1	—	+
Canine papilloma.	Clinically uncommon.	Oral cavity Lips	16–20 16–20	+	Unknown
Mast cell leukemia.	Clinically uncommon.	Visceral peripheral blood leukiemia.	Unknown	+	Unknown
Canine venereal tumor.	Clinically uncommon.	External genitalia of both sexes.	Unknown	+	Unknown

*Invariably fatal.

Canine herpesvirus can sometimes be recovered from canine kidneys which show no gross pathological changes (*9*). Where petechial and ecchymotic hemorrhages are observed, the kidneys should be discarded, since these hemorrhages may result from ICH virus, canine herpesvirus, or leptospira. Since canine herpesvirus has been isolated from the respiratory tract, vaginal tract, fetuses, and nervous system of dogs, this virus must be given special consideration, as herpesviruses are known for their characteristic latency.

Reovirus I persisted in the spleens of germfree dogs or specific pathogen-free dogs for 3½ weeks after intranasal or intraperitoneal inoculation (*13*). Because this virus has been recovered from dogs with respiratory and enteric disease, its consideration is paramount, since it also is a human pathogen. The isolation of ECHO type 6 from the rectal swabs of healthy beagles and the production of enteric disease in experimental puppies emphasizes the importance of this viral agent which presumably is capable of producing disease in both humans and dogs (*17*). It has been shown in experiments with dogs that the virus can persist in the feces for 5 weeks, whereas clinical signs last 3 weeks at most (*17*). Curiously, experimental dogs developed no neutralizing antibodies. No information exists on the persistence of parainfluenza or mumps viruses in the respiratory tract of dogs. There is scant information about the persistence of virus in canine papillomas, but it appears that the warts in any stage are infective; thus, one can conclude that tumor mass contains infective virus, and virus is not demonstrable after the disappearance of tumor mass. The persistence of the infective agent that produces canine mast cell leukemia is unknown at present (*20*). This is also true for the agent that causes the canine venereal tumor which is important historically, since it was the first neoplasm transmitted by tissue transplantation (*23*).

There is great interest in the oncogenic potential of infectious agents. Of the canine agents described in this paper, four have shown evidence of oncogenicity. The Lederle strain 255 of ICH virus produced tumors in hamsters, and the evidence clearly demonstrates that the tumors were produced by this virus (*25*). The other conditions clearly involve the production of tumor masses in the dog—canine papilloma, canine venereal tumor, and mast cell leukemia. The first two tumors usually are self-limiting and the dogs invariably recover. Present experimental and clinical evidence suggests that canine mast cell leukemia is usually fatal (*19*, *20*).

It is very important that this Conference know which canine viral infections are transmissible to humans. There is no evidence that canine distemper or ICH produce disease in man (*3*). We do not yet know about canine herpesvirus, canine parainfluenza virus, canine papilloma virus, canine venereal tumor, and canine mast-cell leukemia virus. The canine papilloma virus is very specific for the oral cavity of the dog, and strict host specificity seems to be characteristic of papillomas. Therefore it is unlikely that dog warts are transmissible to man. The other five viruses—mumps, ECHO virus type 6, reovirus I, pseudorabies, and rabies—produce disease in man.

SUMMARY

The use of primary cell cultures derived from dog tissues for vaccine production obviously presents the medical and veterinary professions

with serious problems. Theoretically, the preparation of human viral vaccines in a heterologous host cell culture probably is attended with less danger from the standpoint of oncogenicity for humans than the use of primary cell cultures derived from primates.

If attenuated virus vaccines for human and veterinary use are produced in cell cultures from dogs, the following procedures should be considered for adoption:

1) Primary cell cultures should be made from specific pathogen-free puppies whose parents are maintained in isolation and monitored for known canine pathogens.
2) Diploid cell lines should be established from various dog tissues of specific pathogen-free puppies for the future development of new virus vaccines for human and veterinary use.

ADDENDUM

Since the paper was prepared, an arbovirus has been isolated from dogs in Singapore (Wilkins, J. H., Bowden, R. S. T., and Wilkinson, G. T.: A new canine disease syndrome. Vet Rec 57–58, 1967). The virus is carried either by ticks or by mosquitoes. It produces a hemorrhagic disease in the dog which may be transmissible to humans.

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Viral Flora of Rabbits

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In considering the viral flora of rabbits, it is necessary to note the particular genus of animal concerned, because marked differences are observed from one genus to another, both in viral susceptibility and in clinical pathology. Eleven viruses associated with various genera of rabbits and hares are presented in table 1. The order in which the agents are listed roughly represents their epidemiologic importance, and the sequence was chosen for convenience in discussion here.

Table 1.—Viral flora of rabbits

Viral group	Name of virus	Natural host genus (genera)
Poxvirus	Myxoma virus	*Sylvilagus* (wild cottontail rabbits in United States and South America). *Oryctolagus* (domestic species in United States and Europe; wild species in Europe and Australia).
	Fibroma virus (Shope fibroma virus)	*Sylvilagus* (wild cottontail rabbits).
	Rabbitpox virus	*Oryctolagus* (laboratory rabbits).
Papovavirus	Rabbit papilloma virus (Shope papilloma virus)	*Sylvilagus* (wild cottontail rabbits).
	Rabbit oral papilloma virus	*Oryctolagus* (domestic rabbits).
	Rabbit-kidney vacuolating virus	*Sylvilagus* (wild cottontail rabbits).
"Myxovirus-like"	Rabbit syncytium virus	*Sylvilagus* (wild cottontail rabbits).
Herpesvirus	Virus III (*Herpesvirus cuniculi*)	*Oryctolagus* (domestic rabbits).
Arbovirus	California encephalitis virus	*Lepus* (snowshoe hare).
Miscellaneous RNA-helical-envelope viruses	Rabies virus	*Lepus* (jackrabbits).
Reovirus	(Antibody; type not reported)	Genus not reported.

Myxoma virus produces a mild endemic disease of wild rabbits (genus *Sylvilagus*) in the Western Hemisphere (*1,2*). Infection is generally manifested by development of a single epidermal tumor, and the virus is transmitted mechanically by mosquitoes and other biting insects feeding through the infected lesions (*2*). In members of the genus *Oryctolagus*, which includes domestic species in the United States and both wild and tame rabbits in Europe and Australia, the virus produces severe generalized infections, characterized by development of widespread subcutaneous gelatinous tumors (*1–3*). Mortality exceeding 99% is observed in susceptible wild populations (*2*).

Fibroma virus in nature infects only wild cottontail rabbits, although domestic species may be infected artificially (*3*). In both cases, localized subcutaneous swellings develop as the result of fibroblastic proliferation (*1*). The lesions persist for 10–15 days after which they regress spontaneously (*1*). The myxoma and fibroma viruses are closely related to one another, as shown by cross-neutralization and complement-fixation tests (*4,5*).

Although immunologically indistinguishable from vaccinia virus, the rabbitpox agent is more virulent than the former for rabbits and is responsible for highly fatal epidemics among laboratory animals (*3*). Nasal and conjunctival discharge and skin rash are characteristic signs of infection (*3*). The disease has not been recognized in wild rabbits (*3*).

Rabbit papilloma virus infects only wild cottontail rabbits under natural conditions, producing benign epidermal tumors that generally regress spontaneously (*1*). The oral papilloma virus, on the other hand, infects only domestic rabbits, producing benign tumors on the floor of the mouth and undersurface of the tongue. The two papilloma viruses also differ immunologically from one another (*1*).

Rabbit-kidney vacuolating virus (RKV) was isolated by Hartley and Rowe from extracts of cutaneous papillomas taken from wild cottontail rabbits (*6*). In structure and chemical properties, the virus belongs to the papovavirus group but differs serologically from Shope papilloma virus, as well as from other members of the group (*6–8*). In cell cultures infected with the virus, cells with vacuolated cytoplasm are observed—hence the name given to the virus (*6*). No pathologic signs have been observed after inoculation of either domestic or cottontail rabbits (*6*). HI antibody does develop, however, and the virus can be recovered from the visceral organs and skin for at least 28 days after subcutaneous inoculation (*6*). Serological tests of cottontail rabbits have shown 28–52% have HI antibody to this virus; no antibody could be detected in the sera of domestic rabbits from several breeding stocks (*6*).

Rabbit syncytium virus (RSV) was isolated by Morris, Saglam, and Bozeman from extracts of the liver and spleen of a wild cottontail rabbit (*9*). Inoculation of monkey and hamster kidney-cell cultures with the agent gives rise to degenerative changes and the formation of syncytia—hence the name proposed for the virus (*9*). No pathologic signs have been observed after inoculation of laboratory or cottontail rabbits with the virus, although CF and neutralizing antibody is produced (*9*). Sera from 8 species of animals trapped in the area where the original rabbit was found were tested for RSV antibody and all were negative except for one-third of the sera from wild cottontail rabbits; no antibody to the agent has been observed in the sera of laboratory rabbits or in human sera tested (*9*).

RSV resembles the myxoviruses in size, nucleic acid type, and in ether and heat sensitivity (*9*). No hemagglutination or hemadsorption has been observed with the virus, and its ultrastructure remains to be described (*9*). The virus differs serologically from the known myxoviruses and from the herpes-, arbo-, reo-, and enteroviruses tested (*9*).

The so-called Virus III of rabbits appears to exist as a latent infection in some stocks of domestic rabbits in the United States and Europe but no isolations of the virus have been reported since 1940 (*1*). The virus does not produce natural disease and species other than rabbits are apparently not susceptible to infection with the virus (*1*).

On the basis of serological evidence, wild rabbits as well as other mammals are frequently infected with strains of California encephalitis virus, and possibly other arboviruses, in nature (*10*). Actual isolation and characterization of one strain of the virus have been accomplished from a snowshoe hare in Montana (*10*).

Like all warm-blooded animals, rabbits are susceptible to rabies virus infection. The agent is listed here as a specific pathogen of rabbits, however, because infections with the virus are endemic in populations of jackrabbits in the northwestern part of the United States, where these animals provide a reservoir of infection for domestic animals and pets.

While actual isolation of reoviruses from rabbits has not yet been reported, as far as we know, reovirus antibody has been demonstrated in rabbit sera, indicating likely experience with these viruses (*11*).

In summary, differences in viral susceptibility and clinical pathology are observed among different genera of rabbits and hares. Of the 11 viruses associated with these animals, 6 appear to infect only wild species under natural conditions. At most, 5 viral agents ordinarily afflict domestic or laboratory rabbits, 2 producing fulminating infections with high mortality (myxoma and rabbitpox viruses) and 2, or at most 3, being responsible only for minor or inapparent infections (oral papilloma virus, Virus III, and possibly reoviruses).

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GENERAL DISCUSSION

Dr. M. M. Sigel: Dr. Wilner, what viruses have so far been found in rabbit kidney cultures, in either primary or established lines?

Dr. B. I. Wilner: In a review of the literature on this subject, all 11 viruses mentioned were isolated from animals. We could find no reference specifically to viruses being isolated in rabbit-kidney cell culture.

Dr. Sigel: Does anyone have information about the incidence of any of these agents or any other agents in rabbit kidney cells?

Dr. R. N. Hull: I can only repeat the negative data which we have reported before. During the past 10 years or so, we have used rather extensively in our laboratory primary rabbit kidney cultures produced from 3-week-old rabbits; we have yet to isolate an adventitious agent from them.

Dr. Sigel: Are the established rabbit kidney cells oncogenic?

Dr. Hull: We have one strain in our laboratory known as LLC RK–1. This strain has been put in suckling rabbits by the following routes: intracutaneous, intravenous, and intramuscular. It has also been put into newborn hamsters. We have at no time observed any oncogenic properties of the strain. It has a near-diploid chromosome number.

Dr. Sigel: Does anyone know if RK–13 is oncogenic?

Dr. J. P. Fox: This is really not relevant to this Conference, but I couldn't help being interested in Dr. Wilner's remark about rabies and the jackrabbits in the Northwest. I would appreciate a little bit more specific information. This was complete news to me. I thought I knew something about rabies.

Dr. Wilner: This information was news to me as well. Unfortunately, I can't give you an actual reference to it. From a discussion with veterinarians from the Idaho and Montana areas, the information was given to me that the virus is endemic. As a matter of fact, at intervals, there are so-called "shoots" in which veterinarians, among others, go out to kill the jackrabbits to eliminate what is considered a reservoir of rabies virus. I asked what happens to the animals after they are shot. And the answer was: "They are left." The animals presumably are left just to disappear in space and the virus along with them.

In a word, I can give you no evidence or data except what unfortunately amounts pretty much to hearsay. I would add, though, as I mentioned at the beginning of the first reference to rabies, jackrabbits as well as other rabbits certainly are susceptible to the virus. And if the agent is in the area, jackrabbits, being wild animals, are potential carriers of it.

Dr. G. D. Hsiung: It is possible for viruses to occur in rabbit-kidney cell cultures without recognition, since the detection of latent viruses in all cultures requires long-term cultivation of the cells. Recently we isolated a herpesvirus, probably cytomegalovirus, from cell cultures derived from horse kidney tissues. Only after prolonged cultivation of the cells, some 80 days and 3 subpassages, was virus-induced CPE obtained. The virus was sensitive to ether, was large, and was morphologically similar to herpesvirus. Since the virus could be propagated only in horse-kidney cell cultures, it was tentatively identified as a horse cytomegalovirus. This example is used merely to show you that recognition of latent viruses in cell cultures requires long-term cultivation. Later I shall discuss in detail the detection of latent viruses in primate tissues.

Dr. P. Gerber: Dr. Kniazeff, perhaps I missed this. How often do you succeed in isolating bovine viruses in fetal calf serum?

Dr. A. J. Kniazeff: The only studies that I know of, where there is specific intent to isolate the viruses directly from serum, are now in progress at the Hyland Laboratory. We know of three definite examples where our own cultures, the characterized bovine cultures which are known to be free of bovine virus diarrhea virus (BVD) were contaminated in other laboratories with BVD when fetal calf serum was used. And the investigators were not working with BVD.

The report by Dr. Malmquist of the National Animal Disease Laboratory also indicates that he and his co-workers have encountered this quite frequently. They are checking just as we do every lot of fetal calf serum. We in our laboratory have not seen BVD in fetal calf serum, but it is probably because we buy large lots of serum and select out of several lots to make sure that it doesn't have BVD in it before we buy it. So I am sure BVD is quite often in the serum.

Dr. J. T. Duff (National Cancer Institute) : In answer to Dr. Sigel's earlier question as to whether or not there have been any viruses isolated from the rabbit kidney, Dr. Johnson at the University of Louisville has informed us that he may have isolated a rabbit virus from some of his rabbit tissue cultures. He is preparing simian foamy virus reagents for us and is using the rabbit kidney. Also, Dr. Enders has sent us a rabbit herpesvirus that has been isolated in his laboratory. I don't recall offhand the name of the person who made the actual isolation. Dr. John Bryans at the University of Kentucky is preparing some horse herpesvirus reagents and feels he may have isolated a rabbit herpesvirus at his laboratory. He has indicated to us that other people have contacted him in regard to isolations of the rabbit herpesvirus from their rabbit colonies.

Dr. P. A. Long (Regional Poultry Research Laboratory, East Lansing, Mich.) : Dr. Kniazeff, have you heard of a case in point at the University of San Francisco Medical Center where the agent for bovine abortion was also isolated in 1965 from a terminal case of pneumonia in man?

Dr. Kniazeff: Which agent was this?

Dr. Long: This is the psitticosis-lymphogranulomatous virus which they term "foothill abortion" on the California coast.

Dr. Kniazeff: I am not familiar with that information. I don't know that it is transmissible to humans. Maybe I didn't understand your question.

Dr. Long: This paper was presented at the University Medical Center. It was preliminary and just identified the agent. But that this man died from pneumonia fits this disease agent that Dr. McKercher is working with—they were synonymous. It was left up to us to find out whether these diseases were the same. I was wondering if anything new had come from that.

Dr. Kniazeff: I do not have any information on that particular case.

Dr. H. Malherbe: In reply to Dr. Sigel's question as to whether agents other than viruses have been found in rabbit kidney cultures, in Johannesburg we have frequently found *Encephalitozoon cuniculi* in cultures of rabbit kidney.

Dr. K. K. Sanford: I wasn't quite sure of Dr. Kniazeff's answer to the previous question. Did you actually find any viral contamination in fetal calf serum? You said there were 3 cultures of bovine origin found to be contaminated that had been grown in laboratories with fetal calf serum. But in your screening of fetal calf serum, have you found any evidence of contamination with this virus?

Dr. Kniazeff: No, Dr. Sanford. I think it really is the same, but I am trying to say that we have not done anything intentionally to try and isolate the viruses. But I think when we speak of contamination of cultures, I will be definite about it. I will say definitely, to make it clear, that virus has been isolated from fetal calf serum in cell cultures of bovine origin which were free of them before.

Dr. Gillespie just pointed out something else too. Of particular interest again is the fact that these isolations can be accomplished from serum known to contain BVD antibody. We have reported sometime back every pool of commercially produced fetal bovine serum that we have tested has appreciable levels of BVD antibody in it, yet you can isolate virus from that serum.

Dr. Sigel: Dr. Kniazeff, what are the chances of finding interferon in bovine serum at a time when an animal may be experiencing an inapparent infection with the virus?

Dr. Kniazeff: Specifically I do not know, but some studies conducted by Dr. Malmquist at the National Animal Disease Laboratory indicate that—I believe, this was with the individual fetal bovine sera—the use of certain sera in cell cultures of bovine origin will produce interference, with such agents as BVD or other agents that are susceptible to interferon.

Dr. J. H. Gillespie: I think it is a very interesting fact with BVD that any virus isolated from a bovine fetus or from fetal calf serum is noncytopathic. So one must be looking for this noncytopathic agent. The other point is that *in utero*, the bovine fetus becomes immunologically competent. We know for a fact that the fetus is competent at 6 months of gestation, and perhaps earlier in fetal life.

Dr. F. Deinhardt: I have one comment and one question. My comment on Dr. Gillespie's presentation is that there were reports in the 1940's of two or three possible transmissions of canine hepatitis virus to man with at least circumstantial evidence.

My question is: If I understood Dr. Gillespie correctly, he stated in his last sentence that there was no doubt that canine tissues would be safer than simian tissues for the production of vaccine. I am wondering if he really has any data to state this categorically.

Dr. Gillespie: In answer to the question, it is just a concept that many individuals have accepted for many years. We have no real evidence to support the concept as it pertains to oncogenic agents, but one usually thinks about host specificity when discussing transmission of viral agents. Until now, at least, it seemed safe to assume that a latent agent usually could be transmitted more readily and with greater ease in the homologous host. And the first question, sir, had to do with infectious canine hepatitis in humans, right? To the best of my knowledge, infectious canine hepatitis virus has not produced infection in humans. I am not familiar with the reference to which you alluded.

Dr. G. Barski: I have a question for Dr. Gillespie concerning the dog cells and viruses. Some years ago, we studied the venereal lymphosarcoma in dogs in France, and we had seen this very astonishing alteration of karyotype in these cells. As you know, the normal dog karyotype shows more than 70 exclusively telocentric chromosomes. In the tumor cells, however, we found, as the Japanese did before in their country, and as it was found here in this country, a karyotype of nearly 50 chromosomes, many of them metacentrics. I think that Dr. Alice Moore and Dr. Miles made a similar observation on an nonrelated dog lymphosarcoma they studied with Dr. Meyers. They found also a very strange karyotype, completely different from the normal.

Have you had any experience with normal dog cell cultures? Do they also tend to modify, during long-term cultures, their karyotype toward this kind of chromosome complement which is very different from the normal karyotype? I mean in normal dog cell cultures or eventually in cultures infected with dog viruses.

Dr. Gillespie: No, I have had no experience.

Dr. Fox: I just wanted to come back to the point that Dr. Deinhardt, I think, was trying to get at which I don't believe was quite specifically answered. There has been a fair amount of experience with canine kidney cultures, but I am not familar with the details. What actually has been the experience in working with these cutures in vaccine production in terms of contaminants that have been detected? This is a little different from listing the number of viruses that are known to infect dogs. Actually, in the case of the monkey, many of the viruses we now know about were first discovered by working with monkey kidney cultures.

Dr. Gillespie: People who have worked a great deal with dog kidney cells know that they occasionally do have a problem with infectious canine hepatitis virus. There is now evidence that canine herpesvirus may also persist in dog kidneys. Considerably less is known about this latter infection because it was just recently reported.

Dr. J. Furth: We have to be guarded with certain conclusions on canine venereal sarcoma. This tumor is transmitted by intercourse in both males and females; but as far as I know, nobody has isolated a virus from it. It is readily transplantable and even "spontaneous" cases have been known to regress.

Also, we have to be guarded in using the term "oncogenic"; *e.g.*, in rabbits, we do not have inbred strains for tumor transplantation and failure of transplantability to heterozygous strains does not mean that the culture is not "oncogenic." Conditioning of the host should be considered and acceptance of the grafted tissue distinguished from tumors induced by virus present in the cultured cells or activated in the host.

Viral Flora of Tissue Sources—Simian and Human [1]

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PROPAGATION of viruses in cell cultures derived from various primate sources, human and nonhuman, has provided the laboratory with an extremely valuable method for the study of this group of organisms. One avenue of investigation developing as a result of this methodology involves the possible use of such cultured viruses as a source of vaccines.

Unfortunately, regardless of the admitted overall value of cell culture, a number of problems have developed as a consequence of the use of cells derived from diversified animal sources. Most important has been the recovery of well over 50 agents from several nonhuman primate materials—mostly from kidney cell cultures, but some also from throat swabs and fecal extracts. It must be emphasized that other animal sources have also provided a host of viruses which will not be discussed at this time. The majority of these viruses were obtained from apparently normal animals. Furthermore, many have not been associated with any overt disease; however, experimental studies with these viruses have been limited.

This report presents the various recognized viruses isolated from primate tissues of human and nonhuman origin. No attempt will be made to review the vast literature concerning the cultivation or characteristics of the agents discussed. Others at this Conference undoubtedly will emphasize the pertinent literature.

SIMIAN VIRUSES

The most frequently used nonhuman primates for virus studies have been the rhesus (*Macaca mulatta*), cynomolgus (*M. irus*), and African

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green (*Cercopithecus aethiops*) monkeys. More recently other simians, both Old World and New World, have been introduced for large-scale virologic studies: to cite a few, patas (*Erythrocebus patas*), marmoset (*Saguinus sp.*), and baboon (*Papio sp.*). It is rapidly becoming apparent that many different species of simians will be added to the present list.

Additions such as these undoubtedly will complicate further the present situation by increasing the number of recognized simian agents. Most necessary will be the recognition and characterization of these new isolates by adequately trained personnel using appropriately prepared reagents and following an orderly system of characterization (*1*).

Table 1 lists the known simian agents grouped according to recommendations put forth by Hull (*2*) and others (*3, 4*). Most viruses were recovered, as indicated above, from tissue culture preparations, primarily kidney cells, although fecal extracts and throat swabs accounted for a number of the isolates. Adenoviruses predominate by far, especially as recovered from tissue culture preparations. These viruses have received the greatest attention because of the oncogenic capability of a relatively large number of the simian adenoviruses: SV20, SV33, SV34, SV37, SV38, and SA7 (*5*). Interestingly, many adenoviruses may be considered as "purely" respiratory tract viruses, *e.g.*, SV1, SV11, SV15, SV17, SV23, and SV32.

The role of adeno-associated viruses (AAV), as described by Atchison (*6*) and found with SV1, SV11, SV15, SV20, SV23, SV25, SV31, SV34, and SV36, will not be discussed (*7, 8*).

Table 1.—Source of isolation of the simian viruses*

ADENOVIRUSES:		
Rhesus and cynomolgus monkeys		African green monkey
SV1—T.C.	SV30—R.S.	SA7—T.C., R.S.
11—T.C.	31—R.S.	17—T.C.
15—T.C., R.S.	32—T.C., R.S.	18—T.C.
17—T.C., R.S.	33—R.S.	V340—R.S., T.S., organs
20—R.S.	34—C.N.S.	Squirrel monkey: R.S.
23—T.C.	36—R.S.	Capuchin monkey: T.C., R.S.
25—T.C.	37—T.C.	Owl monkey: R.S.
27—T.C.	38—C.N.S.	Cinnomon ringtail monkey: R.S.
		Marmoset: R.S.
ENTEROVIRUSES:		
Rhesus and cynomolgus monkeys		
SV2—T.C., R.S.	SV35—C.N.S.	SV47—R.S.
6—T.C., R.S.	42—R.S.	48—R.S.
16—T.C., R.S.	43—R.S.	49—R.S.
18—T.C.	44—R.S.	African green monkey: SA5-R.S.
19—R.S.	45—R.S.	Baboon: A13-R.S.
26—C.N.S.	46—R.S.	Marmoset: R.S., T.S.
UNCLASSIFIED PICORNAVIRUSES:		
Rhesus monkey		African green monkey
SV4—T.C.		SA4—T.C.
28—T.C., C.N.S.		

(See footnote at end of table.)

TABLE 1.—Source of isolation of the simian viruses*—Continued

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REOVIRUSES:

Rhesus monkey
SV12—T.C.
59—T.C., Lung

African green monkey
SA3—T.C.
Marmoset: R.S., T.S.

MYXOVIRUSES:

A. Rhesus monkey
SV5—T.C.
Cynomolgus monkey
SV41—T.C.

B. Variety of primates
(Rh., Cyno., A.G., Chimp, etc.)
Foamy virus types 1 through 7—T.C., T.S.

HERPESVIRUSES:

A. Rhesus and cynomolgus monkeys: B. virus—T.C., organs
African green monkeys: SA8—T.C.
Marmoset, squirrel, and owl monkeys: H. platyrrhinae—T.C., organs
Spider monkey: SMV—organs
B. African green monkey: SA6—T.C., S.G.
SGV (Rowe)—T.C., S.G.
GR2598 (Melnick)—T.C.

PAPOVAVIRUSES:

Rhesus, cynomolgus, patas monkeys: SV40—T.C.
African green monkey: SA12(?)—T.C.

POX VIRUSES:

Rhesus and cynomolgus monkeys: Monkey pox—Skin lesions
Yaba-like—Skin lesions
African green monkeys: Yaba—Tumors

UNCLASSIFIED VIRUSES:

African green monkeys
SA10—Oral Swab
11—R.S.
13—Oral Swab
14—T.C.

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*Source of isolation: T.C. = tissue culture; R.S. = rectal swab; C.N.S. = central nervous system; T.S. = throat swab.

A number of viruses are listed among the adenoviruses that have not been "officially" accepted as prototype viruses. Their inclusion here is primarily to remind investigators of their continual presence in specimens from various primate species. Use of different species undoubtedly will expand these findings. One new isolate (V340) has been described in the literature (*9*, *10*) as associated with disease in the African green monkey and baboon.

Many enterovirus serotypes have also been obtained from simians. These occur primarily in the intestinal tract, although other sites such as the kidney or throat have been found to harbor these agents. One "new" enterovirus is listed, primarily because it was originally isolated from the baboon (*11*, *12*). It is highly probable that studies with other simians also will result in the isolation of additional enteroviruses. Important in enterovirus studies, as least among the simians, is the occurrence of infection without development of antibody on the part of the host. These enteroviruses also are poor antigens, as demonstrated by numerous unsatisfactory attempts to produce homotypic high-titered antibody in various host systems.

Three viruses are included among the unclassified picornaviruses: SV4, SV28, and SA4. As indicated by their designation, two are rhesus isolates and the third is an African green isolate. The three viruses have been found primarily in tissue culture preparations. SV28 also has been recovered from the central nervous system of a rhesus monkey. No isolates of these viruses have been made from the gastrointestinal tract.

Other prototype simian viruses also are grouped to include counterparts of representative human virus "families." The simian myxoviruses currently comprise 9 viruses: SV5, SV41, and the foamy viruses (7 prototypes, possibly more). All of these myxoviruses have been isolated most frequently from simian kidney cell preparations, although other tissues have not been studied extensively. SV5 is of interest because, in addition to being a frequent contaminant of simian cell cultures, it has been recovered from man (*13*) and antibodies to this virus frequently are encountered in man as well (*14*). This virus is related antigenically to certain of the human myxoviruses: mumps and the parainfluenza viruses (*15*, *16*). This cross-reactivity may influence the serologic findings obtained in serum assays. However, Chanock *et al.* (*16*) indicated that the crossing was one way, *i.e.*, SV5 antigen did not react in the CF test with parainfluenza 2 antiserum nor did it cross-react in the HI test. In contrast to the findings with SV5, the findings with SV41 are somewhat different. Originally isolated from cynomolgus monkey kidney preparations (*17*) SV41 is more virulent and found less frequently, at least in rhesus cell preparations, than SV5 (*7*).

Foamy viruses have been found in all simian kidney cell preparations including: rhesus, cynomolgus, African green, squirrel, bush baby, baboon, and the chimpanzee. The extent of species overlapping of the various prototype foamy viruses (7 are recognized at this time) is not known. Serologic testing with various foamy virus prototypes currently is under way in this laboratory and may help answer this question. Presence of these viruses is manifested by the development of cellular vacuolization or lacy appearance, cell syncytia (giant cell formation), and the absence of inclusion bodies. Attempts to isolate these viruses are frequently frustrated by preparations being harvested at improper times. Plummer (*18*) demonstrated that the titer of virus was at its lowest when the cytopathic effect (CPE) was at its maximum.

The use of rabbit kidney cells for cultivation of foamy viruses has increased the ease of virus recovery and decreased the likelihood of involvement with indigenous contaminants. Foamy virus may be present in the throat as well as the kidney, but it has not been found in other tissues or excreta according to Johnston (*19*). He also reported a greater recovery rate of foamy virus by the "unmasking" procedure than by cultivating cells in suspensions (*19*). The SA1 virus originally isolated by Malherbe and Harwin (*20*) is type 1 foamy virus.

Undoubtedly of greatest concern to persons involved with primates or primate tissue are the herpesviruses. B virus (*Herpesvirus simiae*) originally was recovered from a patient with a fatal case by Sabin and Wright in 1934 (*21*). This virus or closely related agents is now known to be present in a variety of simians. It appears that B virus is present primarily in Asian species of monkeys. The African monkeys harbor a closely related but distinct entity referred to as SA8 virus by Malherbe and Harwin (*3*, *20*). Hull (*2*) suggests that SA8 virus in African green monkeys is not as common as herpes B virus is in rhesus monkeys. *Herpesvirus platyrrhinae* (MHV) was isolated from the marmoset by two independent groups of investigators (*22*, *23*), whereas another herpesvirus, temporarily designated as SMV, was recovered from a spider monkey by Lennette (*2*). Much more work remains to be done on this group of agents since little is known concerning their interspecies relationships.

It is generally accepted that only mild or inapparent symptoms occur as a result of infection within the species. Infection of man by *Herpesvirus hominis* is characteristic. The marmoset virus is essentially 100% fatal for that species of monkey, because it is unlikely that this animal is the natural host for this MHV virus. Deinhardt *et al.* (*24*) suggest that this virus is native to the squirrel monkey, since more characteristic lesions are noted in that animal and the marmoset is then only accidentally infected. B virus may be transmitted to various African monkeys apparently not with the same devastating effect as described for the marmoset virus in marmosets (*2*).

No information is available regarding the extent of tissue culture contamination by these herpesviruses. As indicated in table 1, B virus and SA8 have been recovered from cell preparations. Studies on hundreds of baboons and, to a lesser extent, on vervet and rhesus kidney cell preparations in this laboratory only once have indicated a herpes-like virus in baboon preparations. Characterization of this agent is still under study.

Cytomegaloviruses, because of their biologic characteristics, are included with the herpesviruses. SA6 was recovered from African green monkey-kidney preparations by Malherbe and Harwin (*3*, *20*). The other simian isolates (SGV–Rowe; GR2598–Melnick) also have been isolated from African green monkey kidney cell cultures.

Three reoviruses have been found in monkey tissue preparations: two in rhesus (SV12, SV59) and the third (SA3) in vervet. All three reoviruses have been recovered from kidney cell cultures; SV59 was also found in lung tissue. The simian reoviruses are closely related to the human prototypes: SV12 and SA3 to reovirus type 1 and SV59 to reovirus type 2.

One of the more important of the simian viruses is SV40. Not only is this agent the one most frequently found as a contaminant of kidney preparations of various nonhuman primates—in rhesus, cynomolgus, and possibly the patas monkeys—but it is one of the more important of the nonadenoviruses with an oncogenic potential. Infection incidence of this virus in rhesus monkeys has been estimated by Hull to be up to 100% (*2*). The data regarding patas monkey infection are open to question because the possibility of rhesus contact has not been eliminated (*25*). Further evidence of contamination is the fact that the African green has not been found to harbor this virus naturally nor have antibodies been found in sera from newly captured animals (*14, 26*). Furthermore, SV40 antibodies have not been found in other African simians such as chimpanzees and baboons with possibly one exception (*14*). Interestingly, a CPE is not noted in simians where the virus occurs naturally. A typical CPE, however, is seen in kidney cultures from African nonhuman primates (*25*). However, Easton (*27*) indicates that a CPE may be observed in rhesus cells when held for extensive periods.

The oncogenic capability of SV40 for newborn hamsters was first observed by Eddy (*28, 29*). "Transformation" of cells grown in culture by SV40 virus was reported by Shein and Enders (*30*) and by Koprowski and his group (*31*). The findings of Rowe and his collaborators (*32*), with regard to SV40 are disturbing, inasmuch as they indicate hybridization between this virus and an adenovirus resulted in progeny with an SV40 genome within an adenovirus capsid.

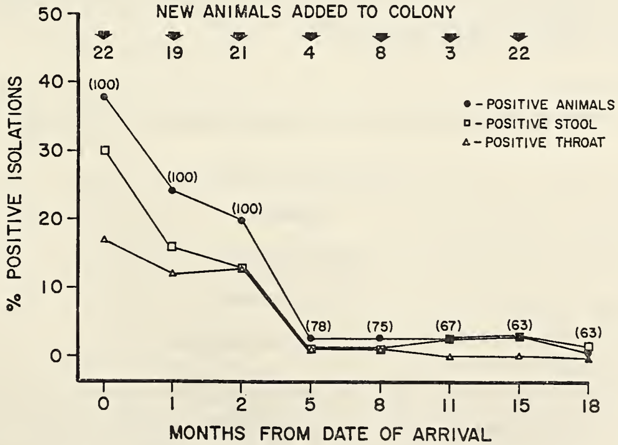
This and other similar reports are of utmost concern to vaccine development and investigators in general. Of some significance, perhaps, has been the limited reports indicating failure to detect overt illness following inoculation or ingestion of SV40; but development of homotypic antibody did occur (*33, 34*).

There are some 5, possibly 6 if SA9 is included, viruses recovered from vervet specimens or tissue cultures that are considered unclassified. Less is known regarding the importance and nature of these agents when compared with other simian viruses. For example, SA11 virus was only infrequently recovered and not from the kidneys (*35*). SA11 virus does show some relationship to another virus (O agent) isolated from mixed washings of cattle and sheep intestines.

Another group of viruses, the poxviruses, should also be mentioned: monkey pox, Yaba, and Yaba-like disease (YLD) virus. It is not known whether these viruses may be found in tissue such as kidney. Their presence as causative agents of monkey diseases is now well documented (*36–38*).

It is evident that the majority of isolates have been found in the rhesus monkey (SV designations). All SA viruses have been isolated from the African green monkey. Some question has been raised concerning the species specificity of these simian viruses. Generally there is a tendency for each virus to be isolated from one species of simian; however, a number of notable exceptions have been reported. The question may be raised concerning contamination of one species by contact with another following capture. Experience and sufficient experimental data are now available to confirm this fact in a number of instances. A classic example of contact infection is the development of measles among captured simians. Data, however, are still necessary with regard to the existence of many of the simian prototypes in nature. For example, SV39 as studied in monkeys freshly captured is not known to occur in African simians. However, this agent has been isolated a number of times from baboons in captivity at our holding facility. Tyrrell *et al.* (*39*) have indicated an outbreak of SV17-induced rhinorrhea and conjunctivitis in patas monkeys. While these investigators claim that the patas monkeys were maintained "under conditions of partial isolation," no definition of this term is given nor does it indicate lack of previous contact with other simians. Generally, one notes an absence of apparent infection among the various simians as a consequence of infection with simian viruses. Serologic tests are usually relied on for laboratory evidence of contact with one or another of these viruses when there is no evidence of virus excretion.

Brief mention should be made at this time in regard to virus shedding. While excretion of virus may not be directly related to the presence of virus in cell cultures of various organs, it does serve as an indicator of the presence of virus either in the respiratory or intestinal tracts. What is in need of study is the relationship between virus shedding and latency, especially as concerns stimulation of latency. Does conversion of an animal from negative shedding to positive indicate a change in the dynamic potential of the animal with activation of the latent state? This is only conjecture, but there are data indicating that "stress" will result in an increased incidence of virus shedding in an otherwise static animal population. As seen in text-figure 1, a baboon population, in which 5–8% of



TEXT-FIGURE 1.—Virus isolations on baseline baboons.

VIRUS VACCINE PRODUCTION

the animals were excreting virus at the time of capture, during shipment to the United States, converted to 40% excreters at the time of arrival. There are a number of factors in need of study concerning this conversion, but the observation has been made also in another laboratory [Kostinkov, *et al.* (*40*)].

HUMAN VIRUSES

Obviously human tissue, because of its limited supply, has not received the attention given that derived from other sources. Human fetal tissue is most frequently used, along with tissues obtained from embryos and infants. Adult human tissue is rarely used for routine purposes, but has served as the source of a number of cell lines now being used in laboratories. In considering viruses of human tissues, it is important to differentiate between a latent infection and infection resulting from transplacental invasion of the fetus during various stages of pregnancy or occurring after birth.

While there is little in the way of published data, there is uniformity of opinion concerning the limited occurrence of contaminating viruses in human tissues. As outlined below, the viruses most frequently encountered are the cytomegaloviruses and rubella. At the same time, it must also be emphasized that these two viruses are very rarely isolated. Other viruses that have been recovered on occasion from nonfetal human tissue are herpesviruses and adenoviruses. To these must be added a long list of viruses reported as recovered, generally as a result of intrauterine infection, from the fetus, stillbirths, newborns, and malformations: rubeola, vaccinia-variola, varicella, poliovirus, coxsackie virus, ECHO virus, mumps, influenza virus, Western encephalitis, lymphocytic choriomeningitis, rabies, infectious hepatitis, and psittacosis. While these may be isolated instances, their occurrence has been reported.

VIRUSES FOUND IN HUMAN TISSUE CULTURE

Cytomegaloviruses

Rubella

Herpesviruses

Adenoviruses

Transplacental Viruses

It is noteworthy that a lack of demonstrable viruses in human diploid cell strains was reported at a World Health Organization meeting a few years ago, as indicated by Hayflick (*41*) and other investigators (*42*).

DISCUSSION

It is well recognized that cells from various animal sources when grown in culture frequently will have signs of the presence of an extraneous agent. Furthermore, these animals may or may not shed in their throat or intestinal tract a virus (or viruses) completely unrelated to the one found in the cell culture. In studies on primates, both human and nonhuman, an indigenous, but possibly not species-specific, viral flora has been described for various species by a number of investigators. Understandably, nonhuman primate sources have received greater attention than human tissues. In most instances the simians contributing the various viruses were not ill. It is known, however, that several of these simian viruses are capable of producing illness and perhaps death. It is not yet clear where and how these simian agents originated in their host.

Of great importance is the source of these extraneous viruses. Present methods of capturing and handling animals used in the laboratory preclude any intelligent pinpointing of virus source. Cross-contamination between the animal under study and other primates strongly influences the results obtained. Present primate holding facilities generally are nothing more than "zoos" in which species are intermingled and attended by common handlers. Certain reports in the literature attributing virus isolations to a particular primate species do not take this factor into consideration. Thus a fair evaluation of what each primate species "normally" harbors is an unknown at this time.

One may speculate on the differences in relative incidence of adventitious viruses in humans and nonhumans. If tissues were obtained from man, held and handled under conditions approximating those used for simians, would this be reflected in the results obtained? In this same vein, it is also of interest that human viruses have been recovered only occasionally from nonhumans. Serologic data, on the other hand, suggest an extremely high incidence of antibody to human viruses or their antigenic relatives among nonhuman primates (*14*). Speculations such as these may, in part, be resolved by epidemiologic and ecologic studies. These studies have been done to only a very limited extent (*14*, *43–45*). Arboviruses are not discussed at this time. Their presence in nonhuman primates as contaminating viruses is in need of study.

Undoubtedly present methods for collecting animal tissue for vaccine production are unsatisfactory. Perhaps partial resolution of this problem may result from development of nonhuman primate fetal tissues having a high degree of sensitivity to human viruses. Preliminary data collected in our laboratory suggest this potential. In essence, development of such a tissue source is essentially that proposed by Hayflick and Moorhead (*46*). The number of baboon and other simian fetal tissues examined at this time is insufficient to allow any definitive statement, but this baboon fetal tissue system offers many of the advantages of human diploid cells and eliminates some of the disadvantages.

However, until more information is available on viruses and virus diseases of primates used for laboratory purposes, a certain reluctance may stem from fear of outbreaks such as that observed recently in Germany where an obscure, fatal disease resulted from human contact with African green monkey tissues (*47*). The possibility of establishing large primate breeding colonies providing stabilized, conditioned, and microbiologically documented animals currently is under consideration.

Demonstration of virus in tissue is not the easiest of occupations. Obvious virus proliferation with development of a cytopathic effect is relatively easy to detect, but it is known that viruses proliferate without overt evidence of their presence. Development of a better methodology would be most helpful for the detection of latent, incomplete, associated, and accessory viruses as well as for elimination of undesirable agents.

Finally, brief mention must be made of the classification of simian viruses. The present system of SV and SA designations being used for Asian and African simians is far from satisfactory. These terms have been useful but will not satisfy future needs, especially since expansion of the use of simians from many different parts of the world is contemplated. It is not within the scope of this paper to discuss the various suggestions for simian virus classification. However, a system approximating that used for human viruses is being developed.

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A Hemorrhagic Disease Transmitted From Monkeys to Man

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THIS is a report of an *ad hoc* committee set up by the Permanent Section of Microbiological Standardization of the International Association of Microbiological Societies.

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In August 1967 a number of persons in Germany who had contact with the blood or organs of a single batch of vervet monkeys became ill with a hemorrhagic disease. Further cases occurred in September in Belgrade. The source of infection has not yet been traced beyond the monkeys, and it was believed that a factual account of the episode should be written so that others handling monkeys may be aware of the dangers involved. This report cannot be complete. It can only give details, some of which may not appear to be pertinent, but until the source of infection has been traced and the infectious agent identified, it is not possible to be selective about the inclusion of the data.

MONKEYS

Transport

During the last 5 years increasing numbers of vervet monkeys have been collected from many areas in Uganda. In July, August, and September of 1967 there were no changes in collecting procedures. Monkeys for export were caught mainly in the Lake Kyoga area and brought to three collecting stations: Namasale, Kidera, and Ndolwa. At suitable times these animals were then taken to Entebbe. On shipment, monkeys were caged singly in compartments in rafts, except for small monkeys which were caged 2 or 3 to a compartment. Monkeys were held at Entebbe for 3 or more days before shipment to Japan, Sweden, Italy, Holland, Switzerland, the United States, Yugoslavia, and Germany. Possibly in the holding station these monkeys were in contact with monkeys caught in other areas of Uganda. Normally these animals would have been transported to Germany and Yugoslavia directly, but due to difficulties caused by the Middle East war since June 16, monkeys were flown to London, where they were held in the RSPCA animal hostel until they were transferred to another plane. Monkeys for Japan, Sweden, and Switzerland, although shipped over the same period from Entebbe as those going to Germany and Belgrade, continued to be sent directly, and no hemorrhagic disease has occurred in the laboratories in these countries. No hemorrhagic disease has occurred among the monkey handlers in Uganda and the disease does not appear to be present in Uganda.

Holding at London Airport

The RSPCA animal hostel handles all species of animals in transit. This holding area is extremely busy and it is not possible to isolate every species or to disinfect the hostel between each shipment of animals. The batches of vervet monkeys associated with the transmission of disease in humans were shipped from Uganda between July 27 and 30, and they were held at the London airport between July 28 and August 1. During this time there were no less than 48 species of animals and birds from all over the world that could have had indirect contact with the vervets. In particular, 1 of 2 langurs (*Presbytis*) being shipped from a Ceylon zoo to Holland, became ill and died on August 1. It may also be significant that the monkeys had the greatest contact with a large variety of birds from many parts of the world; there were also rhesus monkeys in a different room in the hostel.

INFECTION IN MAN FROM MONKEYS

Contact With Monkeys

Within 7 days of arrival of the monkeys at their destination, they were used for cell culture preparations or their brains were removed. The kidneys were carried to the laboratory in petri dishes or centrifuge tubes for processing and the tissue culture containers were sent to the cleaning area; here again the staff had contact with monkey blood or organs.

Monkeys from the shipments of July 28 and 30 could have infected 15 of 20 of the Marburg primary cases, 5 of 6 of the Frankfurt primary cases, and the 1 Belgrade primary case. For the remaining 5 cases in Marburg, contact with an earlier shipment of July 21 was the only possible source of infection, while for the remaining case in Frankfurt a later shipment (August 10) was a highly probable source. All shipments of animals were housed in separate rooms of the same building, with the same air-conditioning at the respective institutions. One infective monkey could explain those 5 cases in Marburg, and by the day of infection this shipment had been kept in "quarantine" for 28 days. If, for the Belgrade case, the shipment of August 1 was the infective one, the monkeys had been "quarantined" for 24 days.

Analysis of Clinical Cases (Table 1)

TABLE 1.—Clinical cases

	Cases	Fatal	Secondary	Doubtful
Marburg	20	5	2	—
Frankfurt	2	2	2	2
Belgrade	1	—	1	—
Total	23	7	5	2

Taking the day of exposure as 0 and not counting the day of onset, the incubation time for 6 of the primary cases ranged from 3–7 days (1 at 3 days, 3 at 6 days, and 2 at 7 days). Five secondary cases occurred 5–8 days after exposure (2 at 5 days, 2 at 7 days, and 1 at 8 days).

Distribution of primary cases.—The cases were distributed by dates as follows: First case in Marburg was on August 8, last case, September 5, with most cases from August 14–27; first case in Frankfurt was August 14, last case, August 23; and Belgrade case was on September 2.

Distribution of secondary cases.—The cases were distributed as follows: Marburg, August 27 and 31; Frankfurt, August 27 and 31; and Belgrade, September 10.

Human exposure and primary cases.—There were 25 primary cases with 7 fatalities. From their occupational hazards the patients can be divided into the following 4 major groups:

Group	*Patients*	*Fatalities*
1) Surgical procedures with monkeys, including removal of kidneys and brain, opening of thoracic and abdominal cavities, autopsies, and taking blood samples from the monkeys inside their quarters	20	5
2) Work with monkey organs outside animal quarters, such as trypsinization and mincing of kidneys	1	1
3) Cleaning of tissue culture containers	3	1
4) Accident, broken tube	1	0

Human exposure and secondary cases.—There were 5 secondary cases and no fatalities. All personnel, performing the surgical procedures and doing the work with monkey organs outside animal quarters (as mentioned above), were protected with gloves and masks. They did not, however, wear them after they finished nephrectomies, autopsies, and mincing, *i.e.*, when they were doing such work as packing cadavers into plastic bags for the incinerator and cleaning rooms and instruments, etc. Animal attendants who were not in contact with blood were not infected, nor were the personnel who took the usual precautions when working with viruses.

Clinical Picture in Man

The primary symptoms were: sudden onset of conjunctivitis, headache and backache, prostration, vomiting, and diarrhea, with high temperatures up to 40°C. A nonhemorrhagic rash (exanthema scarlatiniform or morbilliform) occurred after a few days, together with an enanthema which was frequently hemorrhagic. The scrotum or labia were edematous. There were hemorrhages in the gastrointestinal tract and lungs, including epistaxis, and a strong tendency for bleeding after injections. Anuria and myocarditis were symptoms. There was no icterus. Three deaths occurred on day 8 and one death on days 9, 15, 16, and 17.

The early stage was characterized by elevated transaminases (serumglutamic oxaloacetic transaminase and serumglutamic pyruvic transaminase) to extreme levels. The degree of uremia and creatinemia depended on kidney involvement.

Blood count indicated marked leukopenia, with a shift to the left, and marked thrombocytopenia. There was consumptive coagulopathia.

Pathology in Man

Liver: disseminated necrosis, central and peripheral, of groups of cells or single cells, moderate fatty degeneration, inflammatory periportal infiltration.
Spleen: wide destruction of red pulp, follicular necrosis.
Kidney: tubular insufficiency, degeneration of tubular epithelium.
Lung: hemorrhagic pneumonia (2 cases).
Heart: myocardial opacity, dilation of both ventricles.
Brain: moist swelling.
Testes: frequent hemorrhages.
Skin: bullous exanthema and necrosis.

LABORATORY INVESTIGATIONS OF INFECTIOUS AGENT

Attempts at Isolation

The infectious agent was isolated successfully from blood and organ suspensions in Marburg, Frankfurt, and in the M.R.E. in Porton, England.

Postmortem material and acute blood (or serum) samples from several cases were inoculated into embryonated eggs by the yolk-sac, allantoic-cavity, and chorioallantoic-membrane route. Material from the third yolk-sac passage was negative when tested in guinea pigs as described below.

Similar materials were cultured for bacteria including leptospira and pasteurella; all gave negative results.

A variety of cell culture systems have been used for attempted isolations. These include the cell lines LLC–MK2, HeLa, L, BHK–21, HEp–2, and permanent cells from porcine kidney, human goiter, cercopithecus kidney, and cercopithecus spleen. Primary cells from chick embroys, calf testicles, baby guinea pig kidneys, baby hamster kidneys, and cercopithecus kidneys also were used, as well as human diploid cells (WI–38). No clear cytopathogenic effect was observed in these cells (partially over 3 passages).

Tissue cultures prepared from the kidneys of monkeys (probably infected) were also tested for the presence of the infectious agent. The supernatant of such a culture harvested on day 21 after the cells were seeded (after 3 medium changes, theoretical dilution factor of the cell suspension 1:5,000,000) produced pyrexia in guinea pigs 4 days after inoculation.

Guinea Pigs

Guinea pigs inoculated intraperitoneally (ip) with original material from patients (except from the two doubtful cases from Fankfurt) became febrile after incubation periods ranging from 4–10 days. The guinea pigs remained febrile for up to 6 days but, although they failed to thrive, only a few died. Blood from patients was infective for guinea pigs in dilutions up to 1:10,000. Whole heparinized or lysed blood taken during the febrile stage and from organ suspensions from feverish guinea pigs were successfully passaged both ip and intracutaneously (ic) in guinea pigs through 10 passages. Pyrexia was produced by dilutions of guinea pig blood up to 1:1,000,000. The incubation period shortened to 3 days and most guinea pigs died from 1–9 days after inoculation.

At autopsy, affected guinea pigs were found to have marked splenomegaly, variable degrees of lung consolidation with and without pleural effusion, occasional hemorrhages in the kidneys, congestion, and, in the late stages, macroscopic areas of apparent degeneration of the liver. The testes were occasionally enlarged. No scrotal reactions, typical of rickettsial infections, were seen. The blood of moribund guinea pigs failed to clot.

Pathology in Guinea Pigs

Histopathology in guinea pigs is described in some detail, since this might be useful for all investigators attempting to screen materials for the agent.

Brain.—Irrespective of route of inoculation and guinea pig passage, the only changes observed were in the vascular system. Small venules and capillaries had hyperplastic endothelial cells, whereas the lumina were packed with red blood cells (RBC), with occasional macrophages, or were filled with coagulated homogeneous albuminous material.

Lung.—Only slight interstitial thickening of capillary walls and an increase in the number of mononuclear cells were observed in the first passage, but in the second and third passages a diffuse interstitial pneumonitis without any peribronchial lymphocytic infiltration was observed. The most striking changes were in the capillary walls, with cuboidal metaplasia of cells and increasing numbers of free macrophages within the alveoli. Many macrophages appeared foamy and contained in their cytoplasm partly digested RBC, debris of leukocytes, and other cellular elements. In three cases the bronchi were filled with blood and desquamated bronchial epithelium. Most of the arteries were tightly closed while some veins contained masses of coagulated blood.

Spleen.—In guinea pigs destroyed at a comparatively early stage of the disease, the red pulp of the spleen was a great mass of proliferating reticuloendothelial (RE) cells in the first or second passage. There were also great hyperemia, with large numbers of macrophages containing various cellular debris, and masses of cellular debris lying free within the spleen sinuses. The lymphoid tissue was affected little, although the number of cells was reduced slightly and immature lymphoid forms tended to appear. In all the spleens a few normoblasts could be found. The spleens of the second and third passages from animals destroyed *in extremis* presented a different picture. The amount of lymphoid tissue was, as a rule, greatly reduced and many cells were large lymphoblasts. The red pulp was packed with RBC and coagulated serous material and contained large masses of degenerating leukocytes and other cell debris. The number of RE cells was greatly reduced, for many of them underwent degeneration. The remaining RE cells were greatly hyperplastic, about 3 times the normal size. The nuclei of some of these cells were seen to divide and form clusters of megakarocytic cells. In addition to these cells, many macrophages packed with digested RBC were present. In 3 cases there were many megakaryocytes which were actually budding, and division of nuclei of RE cells forming the megakaryocytes could be seen.

Liver.—In the first passage no obvious degenerative or inflammatory changes could be observed. However, scattered throughout the liver were single cells or groups of 2 or 3 cells containing different amounts of granules. These granules were either clumped and filled the whole cytoplasm or appeared as small, slightly elongated structures. The latter were either discrete and sharply defined or arranged in clumps or ringlets. Generally they were basophilic and stained a dark purple with hematoxylin and eosin, whereas they stained reddish-purple with Giemsa. With Macchiavello's method, the granules stained bright red. Feulgen preparations revealed similarly situated, positive material in the cytoplasm of some liver cells. The granules were also periodic acid-Schiff (PAS)-positive and stained brown with von Kossa's method. After sections were treated with normal hydrochloric acid for 10 minutes at 60°C, they gave negative results with von Kossa's reaction, but when the same sections were counterstained with hematoxylin and eosin the granules were undamaged within the cells and stained a dark purple. In cells packed with many granules there appeared to be a gradual deposition of calcium salts around the minute structures, resulting in a complete obliteration of the cell by an amorphous mass.

In guinea pig cultures of the third and fourth passage, very small focal necrotic lesions could be found in the livers. In some livers, however, these necrotic areas were confluent and formed sharply circumscribed areas. In two guinea pigs the center of the necrotic mass was filled with coagulated blood. As a rule no granules could be found in the center of these necrotic areas, but comparatively large numbers of granules were found in cells at their periphery. In cells undergoing early degeneration without nuclear changes, the cytoplasm appeared to contain small numbers of discrete granules only.

Testes.—In two guinea pig cultures from the third passage, the epididymis contained many RBC mixed with the testicular ejaculum.

Rickettsias were looked for extensively in impression smears of various tissues stained with Giemsa, Macchiavello, and nucleic acid. None were seen. Cells of such smears, however, contained many pleomorphic bodies giving bright fluorescence when stained with labeled antibodies from patients' sera taken late in the infection. Similarly, in guinea pigs inoculated intracerebrally, cells have been found in the liver containing many intracytoplasmic bodies. These are pleomorphic and resemble rickettsias in staining and morphological characteristics.

Serology

Paired serum samples from patients in Frankfurt and Marburg were examined in a complement fixation (cf) test against a prepared antigen. None of the sera was anticomplementary. Table 2 summarizes the results.

Further Investigations

Passage material has been inoculated into tissue cultures to adapt the agent to animals other than the guinea pig. So far, BHK–21 cells in only one bottle have shown any signs of degeneration after 6 days at 37°C. Tissue culture fluid from this bottle was inoculated into guinea pigs, and these guinea pigs became febrile 4 days after inoculation. Further tissue culture lines [primary rhesus and patas monkey kidney, 3 vervet monkey kidney lines, KB cells, Chinese hamster kidney, a continuous rhesus monkey embryonic kidney line (MA–104), and human diploid WI–38] also are being tried.

Table 2.—Complement fixation test titers of patients' sera

Location and name of patient	Serum specimen		Reciprocal CF titer
	Date (1967)	Day of disease	
Frankfurt:			
Hartz	8/24	2	4
	9/21	30	32
Flak	8/25	11	8
	9/21	39	16
May	8/30	4	4
	9/21	25	32
Giesel	9/1	2	4
	9/21	22	32
Marburg:			
Fritsche	8/21	13	32
Kraft	8/25	11	64
	9/27	43	32
Diener	8/25	11	16
	9/27	43	32
Muller	8/25	9	16
	9/27	41	64
Control guinea pig immune serum			32

Sera from convalescent febrile guinea pigs and patients have been tested by Dr. Macrae, Colindale, London, England, against rickettsial pox, typhus, and Rocky Mountain spotted fever, Q-fever, herpes simplex, measles, and psittacosis-lymphogranuloma group antigens in a CF test. All were negative at a dilution of 1:5. The Weil-Felix OXK agglutination test was also negative.

Sera from convalescent (19 day) febrile guinea pigs were examined in a hemagglutination-inhibition test against Semliki Forest, Sindbis, Chikungunya, Japanese encephalitis, Dengue 1, Dengue 2, Tembusu, Langat, West Nile, Yellow Fever, Louping ill, Bunyamwera, and Tahyna antigens; all were negative. Dr. Casals (Yale Arbovirus Unit, New Haven, Conn.) has tested these sera against a wide series of arboviruses but found no antibodies. Dr. Williams (Entebbe) has tested several immune guinea pig sera against further arbovirus antigens but also found no antibodies.

Infected guinea pigs were treated with terramycin and chloramphenicol in a variety of experiments. Some guinea pigs were infected after a week's course of antibiotics, the antibiotics being continued after experimental infection; some received antibiotics starting at the time of infection while others had antibiotic treatment when they became febrile. Neither antibiotic prolonged the illness, although several treated guinea pigs remained afebrile. Antibiotic-treated, control guinea pigs remained well.

Since the most probable route of infection of the human patients was through the skin, 2 guinea pigs were shaved and left for 72 hours to allow any minute skin lesions to heal. Infected guinea pig blood was then smeared onto the skin and allowed to dry. One of these guinea pigs became febrile 10 days later; the other has remained well.

Two *Cercopithecus aethiops* monkeys have been successfully infected with infected guinea pig blood (ninth passage) inoculated ip (0.5 ml). The agent was detected in the blood of both monkeys on the 2d day after infection, and the monkeys died 6 and 7 days after infection. It is too early to report on any other findings.

The agent passes through a gradocol membrane of 790 mμ average pore diameter (APD) but not through a membrane of 340 mμ APD.

Mice and hamsters inoculated with original material have shown no signs of illness. Hamsters inoculated ip and ic with fifth passage guinea pig blood did become sick 5–10 days after infection. Further passage is being carried out. Two suckling (1-day-old) mice inoculated with third guinea pig passage material became sick 9 days after infection and their brains were passaged successfully in other 2-day-old mice. Histological sections from these mice have not yet been examined.

Nothing has been grown in any of the eggs inoculated with original or guinea pig passage material. Fluid removed from L–cell and BHK–21 cultures after 9 days' incubation at 37°C produced illness in guinea pigs when inoculated ip.

Investigations Carried Out in Other Laboratories

Serological studies on patients' sera have been carried out at South African Institute for Medical Research, Johannesburg (Dr. McIntosh); Yale Arbovirus Research Unit, New Haven, Connecticut (Dr. Casals); University of California, Los Angeles, California, (Dr. Work); Middle American Research Unit, Panama (Dr. Johnson); Tropen-Institut, Amsterdam (Dr. de Roever); Robert Koch-Institut, Berlin (Dr. Voss).

Dr. McIntosh reported that some of the patients' sera contained low levels of Group B arbovirus antibodies. This has not been confirmed in any other laboratory, but some patients had received sera from persons recently vaccinated against yellow fever. No significant findings have been made by any other investigator.

Attempts to isolate an agent from monkey and human tissues have also been made by Dr. Bres (Pasteur Institute, Dakar, Senegal, W. Africa). No isolates have been made. Dr. Malherbe (South African Institute for Medical Research, Johannesburg) has reported the occurrence of inclusion bodies after 17 days' incubation in vervet monkey tissue culture inoculated with acute serum from a German patient.

Professor Siegert (Institute of Hygiene, Marburg) has also successfully isolated and passaged an agent in guinea pigs and has demonstrated neutralizing antibodies in patients' sera. He has prepared fluorescent antibody and demonstrated fluorescence in infected guinea pig spleens.

PREVENTION OF FURTHER EPISODES

Without knowing the origin of the infectious agent or its identification it is not possible to be definite about advice that may be given to prevent further episodes. A few general principles can be mentioned which will be useful to all personnel in laboratories handling monkeys.

1) Special arrangements should be made to ensure that different monkey species and other species do not come into contact during shipment. Ideally, monkeys, even of the same species, should not have contact if they come from different areas.
2) All monkeys, from all parts of the world, should be regarded as potentially infectious animals.
3) All monkeys should be housed not more than two to each cage.
4) Different monkey species should not be housed in the same room, even though they are in separate cages or in separate rooms connected by the same air-conditioning system.
5) Rooms and cages must be sterilized between arrival of different batches of monkeys.
6) Unless there are overriding reasons for handling monkeys within a few days of receipt, all monkeys should be quarantined for at least 6 weeks before handling. Contact between monkeys and man during the quarantine period should be kept to a minimum.
7) Protective clothing, including a face shield and rubber gloves, should be worn when monkeys are handled, surgical instruments are used, or cadavers are placed in bags for incineration.
8) Rubber gloves should be worn for all surgical manipulations and the arms should be adequately covered. Rubber gloves should also be worn when material contaminated with monkey blood is handled.
9) Personnel not coming into contact with monkeys but handling the organs must also treat the tissues as potentially infectious.

SUMMARY

A hemorrhagic disease transmitted from vervet monkeys to man, causing 7 fatalities in 27 cases, has been described. The infectious agent survives at 37°C in blood clots but is easily separated from red cells by washing. It will survive for at least 7 days in the refrigerator, will survive trypsiniza-

tion, and probably will multiply in tissue cultures prepared from infected monkeys.

There is no evidence that this episode may be confined to vervet monkeys, and the same disease may be transmitted from any monkey species. Although vervet monkeys have been used experimentally in Uganda for 20 years, there has been no transmission of infection to laboratory workers. No single part of a sequence of events can be singled out as the cause of this episode, but the significant features may be: 1) The route of transport of the vervet monkeys was changed. 2) The monkeys were in contact with other animal species from different parts of the world. 3) The monkeys were nephrectomized and necropsied shortly after they were received. 4) The infected personnel had contact with organs, blood, or tissue and were wearing protective clothing during surgical manipulations but not during all other procedures with material contaminated with monkey blood.

The infectious agent is transmissible in guinea pigs and after 3 passages will kill them. Regular temperature recordings of inoculated guinea pigs, especially between days 5 and 10 after inoculation, show infection by this agent. It is held back by filtration through gradocol membranes between 340 and 790 mμ APD.

These suggestions have been made in an attempt to prevent recurrence of a similar episode.

Viral Flora of Primate Tissues—Discussion

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My discussion will be limited to a few comments relative to the incidence of simian virus isolations. Table 1 indicates the incidence or frequency of isolation of simian viruses by serotype during two periods of time.

During the 1955–58 period we were importing rhesus monkeys by the thousands directly from India. The animals were housed in gang cages and were used shortly after arrival as a source of kidney tissue. As noted, many viruses were recovered and SV4 led the list with 504 isolations. Viruses isolated less than 20 times are not listed.

Table 1.—Frequency of SV isolations

1955–58		1958–62	
Agent	Frequency	Agent	Frequency
SV4	504	SV28	15
SV12	173	SV23	13
SV28	65	SV5	7
SV11	44	SV17	6
SV15	42	SV40	5
SV17	41	SA1	4
SV5	32	SV38	3
SV23	25	SV32	2
B virus	20	SV31	2
Others	<5	Others	1

Subsequently, from 1958–62, the number of isolations declined, and there was a difference in the frequency of isolation of serotypes. Fewer monkeys were used during this period and they were handled differently. Some animals were obtained from suppliers rather than through direct importation, and they were housed in individual cages during most of this period. The African green monkey was introduced at this time, and was responsible for the SA1 isolations. SV28 was the most frequent isolate, but SV4, which had been the most common isolate during the earlier period, was not recovered, and, in fact, has not been seen since 1958. SV28, however, is very closely related to SV4. The SV40 isolations were low only because we did not intentionally subculture rhesus kidney cultures to African green monkey kidney cultures.

The experience with simian virus contamination of cultures between 1962 and 1967 is summarized in table 2. The use of rhesus monkeys during this period probably was less than that of African green monkeys. The most frequent isolates were SV5 and SA1 and most of these came from the African monkeys. A number of isolations of SV41, a paramyxovirus, were made from rhesus monkeys, but these were limited to the year 1963. The other viruses listed were isolated only on rare occasions.

Table 2.—SV types isolated 1962–67

SV5	Most frequent.
SA1	
SV41	12 isolations in 1963 only.
SV5A	Very infrequent.
SV16	
SV17	
SV18	
SV23	
SV26	
SV40	
SA5	

We have not had as much experience with the African green monkey as have some of our competitors who have produced commercial vaccines in cultures prepared from this species. Two examples are presented as the extremes in our experience with African green monkeys in respect to recovery of adventitious agents. In 1963, we produced 19 lots of various experimental vaccines in these cultures. The monkeys were not quarantined or housed as required for production of live poliomyelitis vaccine. As is common practice in vaccine production, uninoculated control bottles were held and examined for adventitious agents. Twelve of the 19 lots yielded simian viruses. Of these twelve, 8 were SA1, 2 were SV5, and 2 contained both SV5 and SA1.

More recently, during 1966 and 1967, we produced a number of lots of experimental vaccines in green monkey kidney cultures. The animals used as source of tissue for these lots, however, were held in quarantine for at least 6 weeks before use. In addition, the monkeys were tested serologically for SV5 and SA1 (foamy virus) antibody. SA1 antibody-positive animals were excluded. We were unable, however, to find SV5 antibody-negative monkeys. None of the cell plantings prepared from the quarantined monkeys during 1966 and 1967 were contaminated with simian agents. Other animals used for testing purposes during this same period, but which were not held in isolation, did yield simian viruses.

The principal problem with the use of the African green monkeys has been the presence of SA1 and SV5. These two viruses are at least as prevalent as they are in rhesus monkeys, if not more so. SA3 and SA5 were recovered once or twice, and on several occasions, a very slowly progressing adenovirus-like cytopathic effect was noted. Subpassage was difficult and generally accomplished only by passage of undiluted fluids. Strained preparations revealed intranuclear inclusions of the type seen in cytomegalovirus infections. Although not serologically identified, the agent probably was SA6 or a related virus.

Viral Flora of Primate Tissues—Discussion

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SINCE it is not probable that primary cultures of adult human cells will be used for vaccine production on a large scale, human virus contaminants may for practical purposes be taken as those that can occur in fetal or neonatal tissues and in cell lines. In considering contaminants of cell culture systems, one should not neglect agents that may be introduced in serum or other materials used in culture production.

Since herpesvirus and vaccinia virus can pass through the placenta, size is not a limiting factor. It is therefore possible that viruses other than those already known to cause overt disease of the fetus will, in time, be found in fetal tissues. Techniques for identifying rubella virus have only recently been developed, and further technical advances are needed before the presence of hepatitis viruses can be detected. The infective forms of cytomegalovirus, vaccinia virus, and herpesvirus can be revealed by current procedures, but it is possible that occult forms of these and other viruses may exist. In addition, the full range of viruses to which a fetus may become tolerant is not known.

It is clear that techniques more refined than those generally used in vaccine safety-testing must be used if we are to detect noncytopathic viruses, occult viruses, or viral components incorporated in the genetic material of the cell. These techniques must include electron microscopy, as well as immunofluorescence where suitable antisera can be prepared. Tests carried out on control cultures present fewer difficulties than those performed on the vaccine itself. If the neutralization test continues to be the mainstay for revealing background viruses in vaccines, the hyperimmune serum must not only be capable of neutralizing the vaccine virus, but it must also be *not* capable of neutralizing possible viral contaminants; this requirement is not generally enforced today. Methods of producing highly specific immune serum, together with other ways of separating by chemical or physical means the vaccine virus from its possible contaminants, deserve greater attention.

In short, if human cells are to be used for vaccine production, intensive efforts should now be directed toward techniques for detecting noncytopathic human viruses and virus-induced genetic changes or antigens, as well as toward improved techniques for disclosing background viruses in the vaccine itself.

Such advances in vaccine control should be applied also to vaccines produced in nonhuman cell systems.

Tissue and Medium Antigens in Vaccines

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CHILDREN are immunized routinely to 6 diseases, and 12 additional vaccines are recommended under special circumstances. Consideration is now being given to the use of vaccines to prevent a larger number of diseases (*1*). Probably over 100 virus diseases are worthy of control when techniques are perfected for production of safe and effective vaccines. For example, there are 58 ECHO, Reo, and coxsackie viruses; 31 adenoviruses, trachoma, varicella, rubella, mumps, arboviruses, parainfluenza, and respiratory syncytial viruses; and at least 54 rhinoviruses (*2*).

We must, therefore, look at the debit side of vaccines and consider their possible harmful effects, including hypersensitization to adventitious antigens, introduction of unknown viruses, and oncogenic potential. Alternatives to vaccine prophylaxis are attractive in some instances, such as control with insecticides of louse-borne epidemic typhus, the use of chemotheraupeutic agents against sporadic rickettsial infections, *e.g.*, Rocky Mountain spotted fever, and the prospect of chemoprophylaxis or therapy of true virus infections, *e.g.*, smallpox.

Many vaccines in common use today may contain foreign adventitious antigens because they were licensed and approved in an era when more sophisticated techniques had not been developed. The use of cell cultures as a source of vaccine production is a tremendous advance over the use of calf lymph, rabbit brain emulsion, yolk sac, or chick embryo. Further application of cell culture technique will permit us some day to reach the ultimate goals for an ideal vaccine postulated by Ikic (*3*), "standardized and certified cell cultures for virus growth, standardized growth and maintenance media, standardized and certified seed virus, and a handling procedure that will exclude the possibility of contamination."

TISSUE ANTIGENS

There is no need to review each of the crude vaccines used in the past because the salient defects can be illustrated with the egg vaccines. The incidence of serious allergenicity to vaccines produced in embryonated eggs is estimated to be 0.5% of the general population (*4*). Stull (*5*) reported in 1946 that 12% of the protein-nitrogen of typhus vaccine was ovalbumin. The vaccine has been improved, and Cox reported that washed rickettsial suspension gave no skin test reactions in subjects sensitized with serum from egg-sensitive persons (*6*). Cohen and Mines (*7*) did immunodiffusion tests of 14 virus and rickettsial vaccines prepared from various portions of the developing chick embryo. They found multiple precipitin bands for various egg proteins in 12 of 14 vaccines, with a marked variation between different products. Other studies by the more sensitive passive transfer test showed marked variation in the amount of egg-white antigen in influenza vaccines from different commercial suppliers (*8*). Influenza virus grown in allantoic fluid and concentrated by the Sharples centrifugation method contained up to 1,000-fold less egg protein than similar virus concentrated by the red blood cell elution method. The 2 influenza vaccines reported negative for egg antigens by Cohen and Mines (*7*) were prepared by the Sharples technique. These 2 vaccines also were positive for egg protein, however, by the more sensitive passive transfer test, and the authors conclude that it is hazardous to use any egg vaccine in an egg-sensitive individual. This has been a standard precaution in the use of egg vaccines for the past 25 years.

More highly purified influenza vaccines have been prepared in recent years and used in extensive clinical trials without evidence of allergic sensitization to egg proteins, even when combined with adjuvants (*9–11*). The prospect of using only the isolated hemagglutinin antigen of influenza virus holds the prospect of retaining only the essential immunizing antigen in the vaccine and at the same time removing the viral antigens which cause febrile reactions in young children but contribute nothing to immunity (*12, 13*).

Blood group substances may appear in many products used in microbiological culture media including peptone, neopeptone, and enzymic extracts of mammalian tissue (*14*).

A blood group A substance is present in White Leghorn embryos and also in egg-grown influenza vaccine (*15*), and this should be eliminated from the finished vaccine regardless of whether it is an integral part of the virus or a contaminant from the host cell.

All the egg-grown vaccines mentioned above were propagated in a manner which insures the presence of extraneous proteins from the culture system. The use of chick cell cultures as a substrate for growing viruses is a great advance, and we have evidence that myxovirus vaccines prepared from chick-fibroblast cell cultures do not contain sufficient egg protein to cause a reaction in egg-sensitive individuals (*16*). Twenty-two children severely allergic to eggs were given live, attenuated measles virus vaccine grown in chick-fibroblast cell cultures and had no unusual reactions. Apparently, chick fibroblasts which have gone through the vaccine manufacturing process do not contain the specific antigens associated with allergy to eggs, chickens, or feathers.

However, another type of allergic reaction apparently not related to egg proteins has been reported. An Arthus-like reaction has been observed in a small percentage of children who received parenteral live measles vaccine after previous injection with inactivated measles virus vaccine (*17*, *18*). The mechanism of the reaction is not clear. Myxoviruses have been shown to become firmly attached to host cell tissue fragments when liberated from host cells (*19*). Others have shown the development of delayed dermal hypersensitivity to live measles vaccine in over 50% of subjects who had previously received alum-precipitated, inactivated measles vaccine (*20*). The sensitizing vaccine (inactivated measles vaccine) was produced in monkey cell cultures, and the live, attenuated vaccine used in the skin test was produced in chick fibroblasts; so it seems unlikely that the sensitization was due to host cell antigens. This conclusion is also supported by the fact that the Arthus-type reaction occurs after natural wild measles following previous vaccination with killed virus. In these cases, it is hard to visualize the minute quantity of host cell antigen attached to the infectious dose of virus as being the cause of the Arthus reaction a week or more later. The nature of the responsible antigen in the inactivated virus and the host response remains to be explained. It seems to be an abnormal host reaction to the viral antigen itself, and such reactions may be more frequently recognized when pure viral antigens become available. Erythema multiforme has been associated with many diseases and with the administration of many drugs, antibacterials, and vaccines. In the latter category are bacille Calmette Guérin (BCG), smallpox, poliomyelitis, and herpes simplex vaccines. Shelley reported (*21*) that 15% of all cases of erythema multiforme were preceded by an attack of herpes simplex. In one patient he could reproduce such a reaction at will by intradermal injection of formalin-inactivated herpes simplex virus.

Within 1 year after the introduction of 3 injections of prophylactic rabies vaccine for Peace Corps workers, 8 trainees developed immediate, severe gastrointestinal reactions to duck embryo rabies vaccine (*22*). In each case the patient had recently tolerated another egg-grown antigen, yellow fever vaccine, without reaction. Both vaccines are produced in eggs and, presumably, the yellow fever vaccine had augmented prior egg sensitivity with the shock organ in the gastrointestinal tract. This explanation has some validity because no previous gastrointestinal reactions to duck embryo rabies vaccine have been reported in more than 100,000 patients who received the standard 14-day course of duck embryo vaccine.

The most impressive evidence that vaccines produced from tissue culture are free from sensitizing foreign antigens is the experience with polio vaccines (*23*). Inactivated polio vaccine was injected repeatedly into millions of people, including newborns, with a very low incidence of sensitized individuals (*24–27*). Neither have any harmful effects been traced to the SV40 virus which was a contaminant in many of the early batches of Salk vaccine. Millions of people, including newborns, also have received the live oral polio vaccine of the Sabin type without reports of sensitization.

Monkey kidney cells contain blood group antigens closely related to human blood group substances, and it might be anticipated that the finished vaccine would contain these antigens. In extensive studies we were not able to demonstrate their presence nor could we stimulate recall of antibody to Rh antigens by repeated injection of Salk vaccine into patients known to be previously hypersensitized to blood group substances (*28*). Repeated injections of vaccine caused no evidence of kidney damage (*29*). The total protein of finished polio vaccine is extremely small when compared to previously produced crude vaccines (*30*), and we must conclude, on the basis of extensive clinical experience and laboratory tests, that whatever cell antigens remain in the finished product must be weakly antigenic and are not prone to cause hypersensitization in man after repeated parenteral inoculation.

ANTIGENS FROM THE CULTURE MEDIUM

There are 2 sources of potential extraneous antigens in vaccines produced from cell cultures—the cells and the nutrient medium. The basic culture medium is chemically defined with added serum and antibiotics, so the most probable antigenic components of the medium are the serum, the antibiotics, and any chance microbial or cellular contaminants.

SERUM

Severe urticarial reactions occur in over 50% of adults given crude antitoxin produced in horses (*31*). The most allergenic portions of the antitoxin are components other than the active antitoxin (*32*), as shown by the fact that antisera can be digested with proteolytic enzymes that remove most of the allergenic components but leave the antibodies intact (*33–37*). With modern, highly purified horse-serum tetanus antitoxin, the serum sickness rate in adults is reported in the order of 5–6% (*38*). Horse-serum antigens from the medium used in the cultivation of monkey kidney cells for the production of inactivated polio vaccine are not present in significant amounts in the final vaccine. They are removed by the following steps: The growth medium used to develop a good sheet of primary monkey kidney cells contains basic medium plus 2–10% horse or bovine serum. After growth of a confluent sheet of cells, the medium is removed and the cell sheet is washed several times with balanced salt solution to reduce residual serum by a dilution of at least 10^{-9}. The cell sheet is then inoculated with the seed virus and covered with maintenance medium containing no serum. After an adequate period for virus production, the cell sheet and virus are harvested with a proteolytic enzyme which removes the cells from the glass and, incidentally, digests any remaining whole serum proteins. This procedure of multiple washing, plus enzyme digestion, as developed by Salk, does not leave enough whole serum proteins to be detected by sensitive laboratory tests; and field trials have shown that the horse-serum concentration is reduced to a level that does not cause clinical reaction in sensitive individuals. Methods for greater purification of poliovirus have been developed and have received extensive clinical use (*39–42*).

ANTIBIOTICS

Antibiotics must be added to primary monkey kidney cell cultures and other primary cell cultures to prevent chance microbial contamination. One of the most effective antibiotic combinations for this purpose is penicillin and streptomycin. However, a serious complication is the fact that penicillin is probably the most common cause of iatrogenic drug allergy during the last 20 years. In addition to therapeutic use by parenteral and oral routes, significant amounts of penicillin may be absorbed from food such as milk, by inhalation, and from reused syringes. In the early batches of Salk vaccine a significant amount of residual penicillin was present in the final product (*43–45*) and some reactions to it were observed. However, it has been shown that replacement of penicillin with other antibiotics in the tissue culture medium removes the risk of allergic reactions (*46*). If a certified cell culture free from contamination were used for vaccine production, it should be possible to eliminate all use of antibiotics (*47*).

Silk, as a contaminant added during filtration in the manufacture of biologic products, has caused severe, immediate allergic reactions to a variety of biologic products administered by the parenteral route (*48*). Silk filters should be forbidden in the manufacture of biologic products and replaced by glass, Seitz-type nylon, or stainless-steel filters.

CONTAMINATION OF CELL CULTURES

This can take several forms, including 1) contamination by another cell culture which grows with or replaces the original cell culture, or 2) contamination by microorganisms, including yeasts, mold, bacteria, viruses, and *Mycoplasma.* Contamination of a primary cell culture with another cell culture is not detected very often and presumably is infrequent. The risk of contamination increases the more times a cell line is subcultured, especially if subculture is carried out in a laboratory where other cell lines (such as L cells) are handled. Methods for identifying contamination of this type have been standardized by the American Type Culture Collection

Committee and applied very effectively in the banking of certified cell cultures (*49, 50*). Cells derived from different species can be rapidly identified by chromosome analysis, tests for species antigen by complement fixation, fluorescent antibody, cytotoxic inhibition, precipitin, or dye exclusion tests. Differentiation between cell cultures within the same species is not satisfactory by any tests now available, unless the cell has a distinctive chromosome marker. However, Gartler's studies with glucose-6-phosphate dehydrogenase (G6PD) mutants in human cell culture indicate that most established heteroploid human cell lines contain the type A variant of G6PD which is found in some 35% of American Negroes but not in cells from Caucasians (*51, 52*). The HeLa cell culture originated from a Negro and has the type A variant, and this suggests that most of the human heteroploid cell lines in tissue culture laboratories have actually been contaminated with HeLa cells.

Our own studies confirm the observations by Gartler. Of 41 established cell cultures of human origin with heteroploid chromosomes and infinite life history in cell culture studied by us and by 2 other cooperating laboratories of the Cell Culture Collection Committee (*53*), only one cell is non-HeLa-like in its G6PD reaction (table 1). This cell is RPMI–2650, the mucoid cell line isolated from the pleural cavity of a patient with metastatic cancer of the nasal septum. A number of human fibroblast cell lines and diploid cell lines of limited life expectancy were negative for the G6PD type A variant. The enzyme variants are valuable as markers for cell lines because they tend to be permanent markers of the genotype and are not easily altered by culture conditions. A number of other enzymes occur in several variants and it is possible that they may provide reliable identity markers for cell lines within species.

TABLE 1.—Human polyploid epithelium-like cell lines which contain G6PD isoenzyme variant type A

HeLa
HeLa–229
HeLa–S-3
HeLa–8–8
HEp–2
Intestine 407
Minn. EE
J–111
WISH
Girardi heart
Chang liver
W–K–Conj
NCTC 2544
NCTC 3075
AV3
FL
KB
L–132
Tu Wi
Detroit 6
Detroit 6; Cl–12
Detroit–98
Detroit–98S
Detroit–98/AG
Detroit–98/AH–2
Detroit–98/AH–R
RH
Detroit–B–16
Detroit–30A
Detroit–56
Detroit–78
Detroit–B17
Detroit–52
Detroit–98
Detroit–196
Detroit–110B
Detroit–34
Detroit–173
Detroit–501
Detroit–32

Methods for prevention and detection of fungal and bacterial contaminants are well established and effective (*47*). Laminar airflow and filtration through absolute filters can add much to prevent such contamination

(*54–57*). Pyrogens in culture media and intravenous fluids are due to products of microbial growth, and methods for their detection and elimination are required of biologic products licensed by the Division of Biologics Standards of the National Institutes of Health.

The control of contamination by viruses and *Mycoplasma* is not so well established because not all viruses have been cultivated or identified in the laboratory. Those for which tests are available can be identified and eliminated, but unknown agents create a problem. For example, SV40 virus was in many batches of the inactivated polio vaccine when there was no known method of identifying its presence (*58*). This virus was not inactivated as completely as the poliovirus and therefore millions of people received inactivated polio vaccine containing more or less SV40 virus, some of which was living. More live SV40 virus would be present in the live, attenuated polio vaccine but, fortunately, SV40 was detected and eliminated before the live polio vaccine was placed on the market in 1962.

Many adenovirus strains contain SV40 virus which helps to stimulate high yields of adenovirus. Adenovirus strains freed from SV40 virus tend to yield only very low titers and, furthermore, at least 7 of the adenovirus strains are oncogenic in their own right in baby hamsters (types 3, 7, 12, 14, 18, 21, and 31) (*59*). As an alternative method of immunization, living adenovirus type 4 vaccine, grown in human fetal diploid fibroblast cells, has been given to volunteers by the the oral route with a very good serologic response and with no spread of virus to contacts (*60*). Until the oncogenic potential of adenoviruses and vaccines produced from them is clarified, the distribution of adenovirus vaccines has been temporarily discontinued (*58*).

Influenza and yellow fever vaccines made from eggs containing leukosis virus have been given to millions of people for over 20 years. There is no evidence to suggest that any increase in tumor incidence has occurred as a result of these vaccines (*23*). On the other hand, batches of 17D yellow fever vaccine containing hepatitis virus were responsible for many infections among military personnel during World War II. Such occurrences can be avoided in the future by eliminating human serum from the suspending medium.

A hopeful development for the future of egg-grown vaccines is the recent experience with measles vaccine produced in cell culture of chick embryo fibroblasts from flocks rendered free of leukosis virus. This vaccine produced in cell cultures was not only free of leukosis virus but also free from egg proteins, as shown by the absence of reactions in egg-sensitive individuals cited above (*16*). The presence in a cell of a latent virus genome, which may combine with the vaccine virus when it is added to the system, is an argument in favor of using cell cultures derived from embryos rather than from adult animals. The only viruses present in the embryo would be those transmitted vertically, and one could thus automatically eliminate many viruses acquired after birth by using only embryonic tissues for vaccine production. The use of pre-tested embryonic cell lines would help to eliminate such occurrences as *Leptospira* in primary bovine kidney tissue culture (*61*) and rubella virus in strain L cells for over 3 years (*62*). As a corollary to the leukosis-Rous sarcoma virus interdependence, Feldman and coworkers have shown that adenovirus tumor antigen was produced consistently in infected green monkey kidney cells but the synthesis of the adenovirus capsid antigen did not occur in the absence of SV40 helper, which is apparently needed to complete production of infectious adenovirus (*63*, *64*).

Live viruses have been recovered from sera used to feed cell cultures. For example, Kniazeff recovered bovine diarrhea virus from bovine serum used in cell culture (*65*). This is of importance because bovine serum is widely used in tissue culture media. To eliminate such accidental viral contamination carried by serum, the Viral Resources Branch of the National Cancer Institute, in cooperation with the Cell Culture Collection Committee of the American Type Culture Collection, is experimenting with the production of sterile, unfiltered, fetal calf serum subjected to extensive tests so that it can be certified as to quality and freedom from contamination. So far the results are encouraging. It is not yet clear whether electron microscopy should be added to the screening test of such sera. It is pertinent to recall that virus-like particles may be seen by electron microscopy in cell cultures such as the Burkitt lymphoma cell lines and in L cells (*66*). It seems clear that, for ultimate characterization of cell cultures, electron microscopy may be necessary to demonstrate the absence of virus-like particles. So far this is not one of the standard methods of characterizing cell lines; however, in the future, it might become a reasonable requirement if a fully characterized cell culture is to be used for vaccine production for human use.

MYCOPLASMA

Mycoplasma contamination of cell cultures occurs frequently and may originate from the primary mammalian tissue, bone marrow, embryonated eggs, bovine serum, and, mostly, from cross-infection from other contaminated cell cultures handled in the same laboratory (*67–72*). Established laboratory strains are easy to detect by culture, but some strains are much more fastidious in their growth requirements. Primary cell cultures are contaminated less frequently than established cell cultures that have been transferred many times. This is, without question, due to cross-contamination in the laboratory. It can be controlled by rigid adherence to established aseptic techniques and quarantine of new cell cultures brought into the laboratory. As in the prevention of bacterial and fungal contamination of cell lines, the routine use of antibiotics to prevent *Mycoplasma* contamination is a 2-edged sword. It will prevent many contaminants from getting established in a cell culture, but this apparent protection encourages poor technique on the part of personnel, which increases the incidence of contamination. Eventually such procedures select for resistant strains of microorganisms that propagate in the presence of antibiotics, and they can easily be missed when cultures are transferred frequently with the addition of fresh antibiotics at each transfer.

DIPLOID CELLS FOR VACCINE PRODUCTION

It seems reasonable to continue to exclude the use of heteroploid human cell lines for vaccine production because some of them may be variants of HeLa cells, which originated from an epidermoid carcinoma of the cervix, and may therefore have potential for transmitting some oncogenic property. In the decade 1950–60, the notion gained prominence that serial propagation of freshly established human cell cultures led, in a percentage of cases, to spontaneous alteration of morphology and the acquisition of neoplastic properties. It now appears that this notion was in error, and many examples of spontaneous transformation of human cells in culture can be explained as contamination with HeLa cells or L cells or some other established heteroploid cell line. Furthermore, there is evidence that cells in primary culture rapidly lose some of their organ-specific antigens (*73*), an added reason for using serially passed diploid cell lines in vaccine production. In view of all these recent developments, *cell cultures*, preferably beyond the first tissue culture passage, should provide the safest substrate for vaccine production. The cells with the most precedence from past use are chick embryo fibroblasts and monkey kidney cells. However, we must also reconsider the suitability of human embryonic diploid cell lines for human vaccine production because they have theoretical and practical advantages over chick and monkey cells.

Recent reviews by Hayflick (*74*) and Ikic (*3*) indicate that human embryonic diploid cell cultures come nearest to satisfying all the desirable characteristics of a substrate for human vaccine production. The chief disadvantages advanced against using these cells are that they may contain: 1) latent human viruses, 2) hepatitis viruses, and 3) an oncogenic principle. The answers to these questions are now fairly convincing. Experimental vaccines have been prepared in diploid cell cultures of WI–38 and administered to over 250,000 patients as vaccines against poliomyelitis, adenovirus 4, rhinoviruses, and rubella. These cells carry no known human virus or other microorganisms as shown by extensive laboratory tests. The clinical trials prove that the cells do not carry hepatitis virus or any unknow human virus which can be detected in volunteers. This field experience also indicates that there are no carcinogenic properties apparent after 4 years of observation.

Against the theoretical disadvantages, none of which are supported by fact, one must balance the many advantages of using the human diploid cell for virus production. For example: 1) They are of embryonic origin and therefore free from many contaminants acquired after birth; 2) they do not harbor any known latent viruses, as do monkey kidney and chick embryo cells; 3) spontaneous alteration has not been known to occur in human embryonic fibroblasts; 4) since they are of homologous origin, they should not sensitize man as readily as cells from another species; 5) they have the widest virus spectrum of any culture system so far described and support growth of some viruses that cannot be grown in any other cell system; 6) they can be tested exhaustively; 7) they can be preserved in large quantity at liquid nitrogen temperature for future use; 8) they can be recovered for mass production of vaccine at will; 9) once the cell line has been proved safe, it can be used for producing a wide variety of viral vaccines; and 10) all the above data permit the "seed virus" concept to be applied to a "seed cell" system.

With a well-characterized seed virus and a well-characterized seed cell system, plus carefully screened culture media, one needs only then to concentrate on aseptic procedures and techniques that will exclude the possibility of contamination, to bring reliability and safety in vaccine production to the high standards envisioned by Dr. Ikic. I will close by adding that absolute filtered air and laminar airflow are just becoming available and do supply the final ingredients—the sterile ambient air in the laboratory environment which has heretofore not been possible.

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Tissue and Medium Antigens in Vaccines—Discussion

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Dr. Coriell has presented so complete a review of our subject, "Tissue and Medium—Antigens in Vaccines," that I am left with few degrees of freedom for discussion.

It is obvious that the three principal components of the production system are: 1) the cell substrate; 2) the fluid in which these cells grow; and 3) antibiotics incorporated into the fluid for contamination control.

Our concern with these components derives from the question as to which, if any, is likely to cause harm when a vaccine produced in such a system is injected into or ingested by a human. Since more vaccines are administered to young children than to adults, there is the additional variable of chronicity of reactions. It may take years to answer these questions. In the meantime, we cannot justifiably be paralyzed into inactivity because of the specters that may be raised as to what might happen 30, 40, or more years later. Biological science being what it is, one has to make judgments at certain stages of accomplishment and act accordingly. In fact, that is why we are here.

I do not think that the antibiotics present a serious problem, because they can be tested rather readily. They certainly should be removed from the final product. The fluid medium is essentially in the same category, although it would be preferable to have no serum in the medium. None should be present in the vaccine as used.

I am not at all certain that degraded serum or synthetic products have been investigated fully in this context. Nonetheless, it is possible to measure residual serum content.

Cells can represent another problem entirely. The sensitization capability of nonhuman cells, I believe, can be satisfactorily tested with the passive transfer technique which Dr. Coriell described so completely for the influenza egg vaccines. Let me add another illustration.

In 1962, a special meeting was convened by the Division of Biologics Standards to consider whether measles vaccine produced in canine kidney tissue cultures should be licensed. One question that was raised was whether such vaccines could be administered safely to persons allergic to dog dander.

Sensitization itself is not a great problem, except for those who are anaphylactically sensitive. Dr. Woodin, a member of our faculty, investigated this question. He obtained serum from 4 individuals who were anaphylactically sensitive to dog dander. Passive transfer tests were then performed. While the controls, beagle dander extract (beagles supplied the kidneys for vaccine production), were markedly positive, no reactions were obtained with the final vaccine or control material made from uninoculated cell cultures or the medium.

I do not know how many children have received this measles vaccine, but I am reasonably certain that the number is in the hundreds of thousands.

Insofar as I am aware, there have been no reports of anaphylactic reactions with measles vaccine. I appreciate that it would be easier to support this technique as being of limited usefulness than to conclude that it is perfect. Nonetheless, this system should continue to be a model for this one aspect of vaccine assessment.

There are, however, two other problems which I am not at all certain that we can answer; nor is it entirely clear to me how we should approach them experimentally. One problem is whether a new antigen (or antigens) may result from the interaction between the virus used for vaccine production and the cellular or serum components (if the latter is included in the medium). This new antigen could be detrimental to the recipient of the vaccine, possibly producing a kind of destructiveness different from that to which we are accustomed. I think that a screening technique can be developed for this, utilizing, perhaps, tagged serum from vaccine recipients. One could, of course, assume that any steps taken to degrade or to remove cellular substances from the final product would, at the same time, inactivate such new antigens.

The other problem is related to the interferons. Are any contained in vaccines and, if so, are they concentrated and precipitated by alum? Can and do they participate in new antigen formation? Is their presence potentially harmful because they induce antibody formation and is this related to the mechanism whereby some killed vaccines (RS, *Mycoplasma*, measles) appear to be detrimental when the natural disease is acquired subsequently?

As you can see, having said that we ought not to be paralyzed into inactivity by invoking farfetched specters, I have proceeded to do that very thing. I would not want to infer that this should hinder us in any way, but rather wish to suggest that we should begin to rule in or eliminate these possibilities rationally, either with new, specific data, or from already available facts.

Blood Group Antigens in Virus Vaccines

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THE presence of blood group substances or alloantigens (*1*) in virus vaccines is considered in this report. The term "alloantigen" is interpreted in the widest sense so as to include all antigens present in some, but not all, members of the same species. The alloantigens discussed include not only the antigens of red blood cells, such as the human ABO antigens, but also transplantation and leukocyte antigens. There are two important aspects of this topic: the evidence for the presence of blood group substances in vaccines, and the possible effects which these substances may have when injected.

SOURCES OF BLOOD GROUP ANTIGEN IN VACCINES

Blood group materials in vaccines may be derived from the tissue from which the virus-rich suspension was obtained. This tissue may be tissue or blood taken directly from the infected animals, or tissue culture cells, and may be either from the isologous species into which the virus vaccine is to be injected, or from a heterologous species. Blood group antigens may also be present in biological substances, such as serum added to the culture medium.

Tissue of the Isologous Species

It is clear that vaccines prepared from tissue taken directly from an animal will almost certainly contain blood group antigens unless these are removed in some way during processing, but it is not possible to be so certain about their presence in tissue cultures. Primary kidney, lung, or skin cultures from a human embryo of blood group A usually contain cells that have blood group A antigen on their surface, but detectable A and B antigens disappear rapidly from human cells during culture. Högman (*2*) found that these antigens could not be detected after the eleventh subculture, and Dawson (*3*) found that the rate at which antigens disappear varies from culture to culture. In cultures from some group A human embryos, antigens could not be detected in the primary culture, whereas in other cultures the A antigen persisted until the sixth to eighth subculture, although by then it was present only in a small proportion of the cells. Both Högman and Dawson used the mixed agglutination technique of Coombs and Bedford (*4*), and it is possible that the phenotypically antigen-negative cells may still have a small amount of antigen not detectable by this technique [for discussion of this problem *see* review by Franks (*5*)]. A and B blood group antigens are not detectable in established human cell lines, or in diploid cell lines such as WI–38 or MRC–1 (*6*). In contrast to the A and B antigens in human cell lines, transplantation and leukocyte antigens persist (*7, 8*). The human MN blood group antigens may be present on cultured human cells [Kelus, Gurner, and Coombs (*9*)], such as the HeLa cell line [*see also* Franks (*10*)].

Although the A and B antigens are not detectable in human cell lines, it is interesting that in certain other species in which the A antigen is an alloantigen, it may persist for considerable periods (*6*). For example, the A antigen may be detected on the cells of the RK13 rabbit cell line after as many as 200 subcultures (*11, 12*).

Tissue Cells of Heterologous Species

It is not generally realized that antigens which are blood group antigens in one species may also be present in another species, sometimes even in unrelated species. In the unrelated species it may be present only in some individuals, that is, it may also be a blood group antigen in this species, or it may be present in all individuals. Some of the most widely distributed antigens of this type are the A and B blood group antigens, which are alloantigens in man, and are also present, not only in many other primates but also erratically throughout the animal kingdom [for a review of the distribution of human blood group antigens in primates *see* Kramp (*13*) and Franks (*14*)]. For example, the A and B antigens are present on the tissue cells of rhesus monkeys, but not on their red blood cells, and it has been shown that these antigens are present not only in primary cultures of rhesus monkey cells but also in established lines such as LLC–MK (*15*).

Biological Fluids Incorporated in the Culture Medium

Many blood group antigens, such as the A and B antigens, may be present in an animal not only as an integral part of the cells but also as soluble materials in the body fluids such as the saliva, and more importantly in the present context, in the serum. Thus human beings who are blood group A usually have detectable amounts of A substance in their sera, particularly if they are "secretors." An alloantigen related in specificity to the human

A antigen occurs in many other species—for example, as an alloantigen in cattle where it is known as J substance and where it is always present in J-positive animals. Antigens of this type are frequently adsorbed onto cultured cells from serum and may be very tenacious, proving difficult to remove (*16*, *17*). It is frequently difficult to detect these substances. For example, soluble human A or B antigens would normally be tested for by an inhibition test, by showing that when the putative A substance is mixed with anti-A it inhibits the agglutination of group A red cells by the anti-A. It has been found empirically that it is not always possible to detect cattle J substance with human anti-A and human A red cells in agglutination inhibition tests, but it is possible to detect it by use of a hemolysin inhibition system with cattle anti-J and sheep R-positive red cells (the sheep R antigen is another alloantigen that is related in specificity to the cattle J and human A antigen). It is not possible to detect cattle J substance even by this more sensitive method, once the cattle serum has been diluted in tissue culture medium. This may suggest that only very small amounts of these substances are present, but it is still possible that they will be capable of stimulating the formation of antibodies. A and B antigens are also present in a wide variety of microorganisms, and the possibility that they may be present in extracts of yeast, etc., should not be ignored.

POSSIBLE EFFECTS OF BLOOD GROUP SUBSTANCES IN VACCINES

Because of the way in which virus vaccines are usually applied (by intramuscular, intradermal, or subcutaneous administration), the possible hazards due to the presence of blood group antigens in the vaccines arise not from the chance of anaphylactic reactions due to already existing allergy,[1] but to the possibility of provoking a new allergic state which may give rise to some undesirable immunologic phenomenon at some future date. The most obvious example is hemolytic disease of the newborn. If a woman who is blood group O receives an injection of a virus vaccine containing blood group A material, she may produce immune anti-A, and if she produces group A babies, they may be affected with hemolytic disease of the newborn. There is at the moment no evidence, or even indication, that this occurs in man. On the other hand, servicemen and others who have received frequent injections of bacterial vaccines and toxoids more often have detectable immune anti-A in their sera than do other members of the population.

There are several considerations which will determine whether this is a real, or only an imaginary, hazard. The most important is the way in which the virus vaccine is processed after the virus-rich preparation is harvested, and this is such a variable process that it is not possible to generalize. Clearly, procedures such as growing the virus in cells in maintenance medium which does not contain serum will tend to reduce the amount of blood group substance present. On the other hand, if virus multiplication leads to cell lysis, it is much more likely that soluble cellular material will be present in the vaccine.

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[1] The term "allergy" is used to describe the state of altered reactivity, whether this involves protection from disease or undesirable effects such as anaphylaxis. The term "immunity" is restricted to its original sense of protection from disease (*18*).

A further consideration is whether the way in which the recipient is immunized may lead to the development of a type of allergic response that in itself precludes the development of phenomena such as hemolytic disease of the newborn. An example of this type of response is the phenomenon of "enhancing" antibody which may be produced in rodents given injections of freeze-dried tissue. Far from producing classical transplantation immunity, enhancing antibody gives a prolonged survival to grafts from unrelated animals. Another way in which the development of antibody due to immunization might be advantageous rather than hazardous is in the production afforded against hemolytic disease of the newborn in man due to anti-D in women who are group O and whose babies are group A. It has been established that hemolytic disease of the newborn due to the anti-D occurs much less frequently in these women than in women in which maternal-fetal ABO incompatibility of this type does not occur, and it has been suggested that the anti-A in these women combines with the fetal red cells which enter her circulation, and that they are then removed to a site in the reticuloendothelial system from which is it difficult for them to provoke the development of anti-D.

As pointed out earlier, there is no definite evidence which suggests that such phenomena occur in man, but there are several instances of untoward results of the administration of vaccines in animals. The most extensively investigated of these is the development of hemolytic disease of the newborn in the pig following the administration of crystal violet swine fever (hog cholera) vaccine to the sow. This vaccine, now no longer used in Britain, was produced from the blood of infected pigs to which crystal violet was added to inactivate the virus. The injection of this vaccine leads to the appearance of antibodies in the sow against pig blood cells and the development of hemolytic disease of the newborn in the piglets (*19*, *20*). Another example is the involvement of equine encephalomyelitis vaccine in hemolytic disease of the newborn in the horse. This disease occurs spontaneously in the horse without the experimental or therapeutic injection of horse tissues in a vaccine, but it seems very likely that the higher incidence of equine hemolytic disease of the newborn in the United States at one time was due to the use of equine encephalomyelitis vaccine (*21*). This vaccine has not been used in Britain, where the incidence of hemolytic disease of the newborn was much lower than in the United States (*22*).

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Mycoplasma and Cell Cultures

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CONTAMINATION of cell cultures with *Mycoplasma* was first reported by Robinson *et al.* (*1*) in 1956. Since then, *Mycoplasma* have been shown to be frequent contaminants of continuous cell cultures (*2–20*, *23*, *25*, *34*, *35*).

The incidence of *Mycoplasma* contamination of uninoculated primary and continuous cell cultures is summarized in table 1. In brief, primary cell culture lots were seldom contaminated, whereas continuous cell lines were frequently contaminated.

In our study (*11*, *12*), primary cell culture lots prepared by the Division of Biologics Standards (DBS) Cell Culture Section were sent to the DBS Section on *Mycoplasma* for examination prior to first feed. During the past 7 years more than 900 primary rhesus and vervet monkey and rabbit-kidney cell culture lots were examined. Only 7 isolations were made (table 1). This is less than 1% contamination of the cell culture lots examined. Each cell lot was prepared from 2 kidneys of one animal. The Russian workers (*14*) reported 3–4% contamination.

TABLE 1.—*Mycoplasma* contamination of uninoculated cell cultures

Cell culture	Number positive / Number tested	Percent contaminated	Reference No.
Primary cells	7/>900	0. 8	(*11*, *12*)
	Not reported	3–4	(*14*)
Continuous cells	94/166	57	(*9*)
	75/165	45	(*11*, *12*)
	45/49	92	(*16*)

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[1] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

On the other hand, we have examined 165 continuous cell lines obtained from more than 20 separate laboratories in the United States and Great Britain. There were 75 isolations, 45% contamination. In other surveys, Pollock *et al.* (*9*) reported 57% contamination and the Russian investigators (*16*) reported 92% contamination. The lowest incidence of contamination in continuous cells was found in cells grown in small volumes and in antibiotic-free media and used for cell culture metabolic studies. The highest incidence of contamination was found in cells used to propagate microorganisms or in cells supplied by cell culture producers. These cells were grown under conditions that provide a high contamination risk. They were produced on a large scale—grown in large volumes and in large numbers of containers.

Contamination was found in continuous cells derived from all animal species tested, including human, monkey, bovine, dog, rabbit, hamster, mouse, rat, frog, and fish tissues.

The recovery and identification of *Mycoplasma* species isolated from uninoculated cell cultures are summarized in table 2. The initial contamination in continuous cells usually occurs during cell propagation. One source of contamination is probably the investigator and his environs, since many of the *Mycoplasma* recovered from animal cells are human oral and genital strains (*2, 6, 11, 13, 17, 18, 21–23, 29, 30, 32*).

Trypsin may be another source of contamination, since swine *Mycoplasma* are common contaminants (*19, 24–28, 30*) and trypsin is prepared

TABLE 2.—Identification of *Mycoplasma* contaminants isolated from uninoculated cell cultures

Mycoplasma species	Normal habitat	Reference No.
M. hominis, type 1	Human genital	(*2, 6, 11, 13, 17, 18, 21*)
M. orale, type 1 (*M. pharyngis*)	Human oral	(*13, 20–23, 30, 32*)
M. salivarium	Human oral	(*29*)
M. arthritidis (*M. hominis*, type 2)	Rat arthritis (human genital)	(*18*)
M. hyorhinis	Swine arthritis	(*19, 24–26, 28, 30*)
M. granularum (*M. laidlawii*)	Swine oral	(*27*)
M. laidlawii	Sewage (animal genital)	(*21, 29–31, 33*)
M. gallisepticum	Avian sinusitis	(*17, 30*)
M. arginini	—	(*30, 34*)

from swine tissue. Nonetheless, our attempts to isolate *Mycoplasma* from small samples of several lots of trypsin were unsuccessful. Basal tissue-culture media and sera supplements were not found to be major sources of contamination at the small volumes tested (*11*).

Contamination can be spread to cells by *Mycoplasma*-contaminated virus suspensions. The virus suspensions used in many research laboratories are commonly found to be contaminated with *Mycoplasma.*

In addition, contamination can be spread by aerosols produced with *Mycoplasma*-contaminated cell cultures which can cross-contaminate other cells prepared in close proximity (*15*).

In summary, we found both pirmary and continuous cell cultures subject to *Mycoplasma* contamination risk, and *Mycoplasma* to be common adventitious agents of cells. Continuous cells were found to be contaminated more frequently than primary cells. It would seem reasonable, therefore, to examine cell cultures used for vaccine production for the presence of *Mycoplasma.* Presently, a test for *Mycoplasma* is required for Measles and Poliomyelitis Virus Vaccine (Public Health Service Publication No. 437, § 73.74a) and the test is now under consideration as a requirement for all cell-culture produced virus vaccines.

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Viral Flora of Tissue Sources and Tissue and Medium Antigens in Vaccines—General Comments

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THE speakers have enlightened us on some of the problems inherent in the use of primary mammalian or avian cell cultures for virus vaccine production. The need for vaccine substrates free of adventitious agents is obvious and has been dramatically emphasized by the recent illnesses associated with cercopithecus monkeys. Primary monkey kidney cell cultures for virus vaccines have been extensively used over the past 10 years. Although the occurrence of adventitious agents in this type of cell culture is well recognized, requirements set forth by the Public Health Service regulations have effectively controlled the safety of these vaccines.

We have reviewed our records on several thousand cercopithecus monkeys whose tissues were used in the production of oral polio vaccine. Several aspects of our experience may be of interest. At one time, adventitious viruses caused us to reject 36% of primary kidney cultures. Of these agents, 98% were syncytial, with foamy virus types II and III most frequently recovered, followed in order by simian measles virus and hemadsorption agents. The isolation of monkeys for 6 weeks reduced the number of simian measles and hemadsorption agents but did not significantly lower the total of cytopathic contaminants in cell cultures, since recoveries of foamy viruses increased over the period of quarantine.

Serological screening solved the problem. The sera of monkeys were tested for complement-fixing antibodies to foamy viruses, and kidney cells were selected for culture only from animals lacking such antibodies. As a result, the rate of rejection dropped to less than 10%.

There is little doubt that diploid cell lines of embryonic human or perhaps monkey origin having the characteristics described by Dr. Coriell could provide advantages in the production of virus vaccines. However, the necessity will still exist for cell cultures with a spectrum of virus sensitivity broad enough to test such vaccines for extraneous agents. It seems reasonable to assume that rhesus and cercopithecus kidney cultures would be included.

Testing and retesting vaccines for extraneous viruses are necessary for safety. Detectable contamination in the test cultures could seriously prolong the lead time necessary to produce vaccines in an epidemic crisis. Several years ago, to guard against the necessity for frequent retesting, we initiated a program of pretesting cell cultures and storing them under liquid nitrogen.

Monkeys are selected on the basis of apparent good health and negative serology to foamy viruses. Following quarantine, their kidneys are removed, trypsinized, and suspended in media containing optimal concentrations of dimethylsulfoxide and bovine serum. The kidney cell suspensions are stored in ampuls at concentrations which would give a 1:1 relationship to Povitsky bottles. Ten percent of a suspension is planted in control bottles for testing, in accordance with Public Health Service requirements for tissue culture safety. Ampuls found to contain unsatisfactory preparations are discarded. Rarely indeed is a virus contaminant detected in the uninoculated cell controls if this system is used.

Frozen, pretested cell cultures have been used successfully for poliovirus vaccines and should be given serious consideration in future virus vaccine programs.

In closing, I would like to share the opinion expressed by Dr. Coriell that carefully monitored, banked cultures carried beyond the first passage may provide the safest vaccines.

Viral Flora of Tissue Sources and Tissue and Medium Antigens in Vaccines—General Comments

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WE heard again the compelling arguments repeatedly advanced in the past by Dr. Hayflick and his colleagues; namely, that human diploid cells as substrate for vaccines are as safe as, or presumably safer than, the currently licensed primary cells. The conservative position delaying their licensure, and adopted earlier by the Division of Biologics Standards, was dictated by a reasonable fear that human diploid-derived vaccines could contain substances of unknown potential for causing harm in some humans when mass populations were at risk. Although we must wait until later to hear the most recently accumulated evidence, it is rational to assume that there has been no significant adverse experience in the large-scale field trials to date. Further requirements, if any, that must be met were not elucidated earlier. In contrast, the design of the program has emphasized the large number of adventitious agents seen in primary cells, and we are led to believe that primary cells may contain a collection of undetected agents of undefined pathogenicity.

Therefore, the impression is gained that use of human diploid cells is considerably safer than the tissues now used for vaccine manufacture. A simple extension of this proposition strongly supports the argument in favor of the complete substitution of human diploid cells for the kind of cells presently licensed. This would ensue if we are convinced that primary cell cultures from simian, avian, and canine tissues possess greater potential for disaster than a well-characterized, intensively studied, and certified human diploid cell line.

The practical consequences of such a decision are not immediately evident. For example, it may well be that human diploid-produced vaccines will continue to be relatively expensive to manufacture due to increased labor costs. But it is time to give serious consideration to all implications of a move to adapt the use of human diploid cells to make all live virus vaccines intended for human immunizations. On the other hand, the manufacture of purified viral antigens rendered free of all nucleic acids may safely permit the use of conventional primary tissues, and eventually, even heteroploid cell lines. That the latter seems technically feasible was well illustrated by Dr. Bachrach's presentation. The long-held dream of extensively purified virus particles and subsequent isolation of specific viral antigenic substances may be nearing reality and this could permit the development of ideally safe and potent vaccines.

Viral Flora of Tissue Sources and Tissue and Medium Antigens in Vaccines—General Comments

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Mountains of evidence, revealed at this Conference and displayed in the literature, have indicated the occurrence of viruses, oncogenic agents, and their antigens and antibodies in so-called normal primate and nonprimate tissues used as sources of cells for the propagation of viruses for human vaccine production. In addition, the tissue cells and the media of propagation furnish antigens which induce antibodies with resultant sensitivities to the host cell constituents. Contamination of an established cell with other lines and even wild cells has occurred with the usual confusion of cause and effect. Stopgap measures have been applied to obtain cells free of objectionable infectious agents by both chemical and physical methods as well as by deliberate selection of certain species cells. Attempts have been made to clean up or purify the finished vaccine; they usually resulted in the production of an unstable, nonpotent product or one too costly to sell. All of these methods have contributed to the relief of the problems but have not solved them.

Future regulations concerning the cells used in the production of human vaccines may require that no agent, recognized as being capable of inducing human infection or tumor, either in an attenuated or inactive form, may be included in the preparation of the market product except that stated on the label as being primary to the biologicals' authorized use. Such a requirement would lead to the use of fetal tissues for sources of cells. These sources would be specific pathogen-free animals, gnotobiotic animals, and humans. Fetal cells from such sources would be the closest to being free of man's and animal's infectious and tumor agents. Most viruses could be adapted to growth in the fetal cells of the various species available, such as the diploid cell line, WI–38.

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[1] U.S. Department of Agriculture.

Germfree animal and human fetal cells available now or in the future must be propagated in a laboratory with filtered sterile incoming air, with laminar flow of the air into the tissue culture laboratory, and without the use of antibiotics. Industry could develop this on a commercial basis, with the cell-producing facility licensed and inspected by the appropriate government agency. In this way the cells of the required type could be certified to the research or manufacturing user as being free of the proscribed agents and antigens. The pristine state of these cells would be monitored by the user's control group who would look for changes in karyotype and for the presence of contaminating agents. Culture media, which has been properly prepared and free of serum where possible and which has been sterility-tested, must be available. The premises and operations of the vaccine manufacturer would be open to inspection with regard to the cells used to propagate the virus as well as for the finished vaccine.

Even with rigid physical and technical control of the original cell line and seed made from it, the number of serial passages of cells permitted for use in the manufacturing process cannot be predicted at this time. A diploid cell line is desirable which is preserved in liquid nitrogen storage for use in the serial propagation of cells for virus production. The use of the cell line might continue as long as there were no changes of karyotypic nature indicating a departure from the original cell characteristics, with no contamination by viruses or other microbial forms including no other cultured cell lines, and as long as the virus produced in the cells retained the necessary attributes to make a potent vaccine. This last statement is necessary since the host cell does have the ability to bring changes, particularly in antigenicity, in the virus grown in it.

GENERAL DISCUSSION

Dr. I. Johnson (Lilly Research Laboratories): During the discussions, there has been one item which seems to me has not been touched on, which I suppose you might consider sort of a quasi-epidemiological comment. It has been my experience in looking for tumors in monkeys, during the times that we were slaughtering many of them, that tumors are very rare. And actually looking at thousands of animals, I think I found a few pregnant ones. One monkey did have a benign fibroid tumor of the uterus and one monkey had adenocarcinoma of the head of the pancreas. I think it is generally felt that monkeys have few spontaneous tumors and are relatively resistant to tumor induction.

Man, on the other hand, is not very free of malignancy, and there is at least some evidence that man is not completely free from, or resistant to, tumor induction.

I was interested in the comments that the oncogenic agents of primate origin are not oncogenic in that species but are usually oncogenic in other species. If we consider the hypothetical primate or human cancer virus, I don't think we can rule out a vertical transmission based on any hard data.

In at least two other species—murine and avian—where there is a high incidence of spontaneous malignancy and evidence of vertical transmission of oncogenic agents, these agents are also oncogenic in other species. This is something which has not been seriously considered so far, and I am not really citing it as an argument against using diploid human cell lines.

If I could use the word "consensus," there would be very few people here who would argue against use of diploid lines. But since we are discussing hypothetical dangers, there is a danger of the vertical transmission of human cancer virus in fetal human diploid cell lines.

On the other hand, I think that many of the safety factors cited for diploid lines really are applicable to almost any continuous cell line. One can freeze them; one can conduct all the safety tests before one uses them and perhaps protect them against contamination.

Dr. H. Montes de Oca (Division of Biologics Standards): Is there a description of any noxious or oncogenic effect in the people who have received the vaccine prepared with primary monkey tissue?

Dr. H. Koprowski: I don't think such a question can be answered unless we would find in the vaccines an agent which caused appearance of an unusual malignancy within a short time after its administration. I don't think you can compare incidence of tumors in slaughtered young monkeys with incidence of cancer in man. I doubt that you could evaluate oncogenicity of vaccines during a long-term follow-up in man. No amount of surveillance would permit any conclusion to be drawn from such an observation.

Dr. R. Kirschstein (Division of Biologics Standards): It should be pointed out, in regard to what Dr. Johnson said, that the monkeys which are used for kidney cell production in this country are 18–36 months of age, which is a very young age, indeed, to have tumors. However, in terms of oncogenic effect in primates, Rous sarcoma virus easily induces tumors in this nonhuman primate.

Dr. J. P. Fox: I have perhaps two questions directed to the matter of antigens in vaccines and I don't know whether to ask them of Dr. Coriell or Dr. Franks, or both. I am thinking particularly of the envelope group of viruses which, in terms of practical experience, would include chiefly the myxoviruses and the inclusion of surface antigens from the cells in these viruses. Is there any evidence for possible antibody response in recipients of myxovirus vaccines to the cell surface antigens?

There is some evidence that I cannot remember specifically about studies of blood-group response in recipients of influenza vaccine that may have something of the same basis. I don't recall that Dr. Franks mentioned this and I wonder whether he has any information.

Dr. L. Coriell: I had some comments on that in my paper, but I cut it out to conserve time.

The crude influenza vaccines we have been using in the past do contain group A blood substance as shown by Springer. The substance also is found in chick embryo. So either the influenza virus has group A substance in it, which has been reported, or else the vaccine carries some of the chick embryo over with it.

And I pointed out there is a lot of chick embryo protein in finished influenza vaccine, and the group A substance probably comes from the chick embryo tissues. This can be eliminated as a significant problem if the influenza virus is grown in a cleaner substrate or is purified. Certainly, the myxoviruses do carry over some of the host protein.

Dr. Fox: This is incorporated during the virus particle formation?

Dr. Coriell: Right. This has been suggested as one of the possible explanations of the reaction to measles vaccine when you immunize the patient with killed vaccine and subsequently give live vaccine; perhaps the patient is sensitized to some protein other than the virus.

I think when you examine that, it really doesn't hold up very well, because if the patient is sensitized by the killed vaccine to some protein from the host cell, then you must explain how the reaction to a subsequent live virus can also be precipated by wild measles virus. Presumably, the infectious dose is a very few particles of virus. And they have to carry enough host antigens attached to a few particles of virus to cause a widespread vasculitis 7, 8, or 10 days later.

I just don't think it is possible. The explanation is not obvious, but I think something happens to the killed measles vaccine that alters it, so it combines with host tissue and produces an antigen-antibody-antigen complex which is stimulated later by introduction of only a portion of the antigen. Another unknown factor is that the killed measles vaccine, produced in monkey kidney and concentrated, does contain quite a lot of nonviral protein as compared to inactivated polio vaccine which is not concentrated. Periarteritis nodosa is a type of reaction.

Dr. D. Franks: I would like to make a further point about the blood-group material in vaccines derived from the human tissues. The A and B antigens disappear quite quickly, as has been shown by several workers, including Dr. Brand and his colleagues (Yunis, Yunis, and Brand, *Blood* 25: 96, 1965). The A and B antigens disappear very quickly from cultures of human cells, but the H antigen from which the A and B are synthesized may persist for a longer time.

This might be important because one might think that certain amounts of A and B are still being synthesized, although one couldn't detect it. However, if one looks at human diploid cell lines such as WI-38 or MRC—1 for H antigen, the precursor, one finds it is completely absent. I would put this forward as another reason for using human diploid cell lines.

Dr. M. M. Sigel: Dr. Kalter, how many of these viruses were actually derived from the monkey tissues and how many were inadvertently picked up through contamination in the laboratory where the cells were under cultivation? What would be an intelligent guess as to such frequency of contamination in your experience with your monkey cells?

Dr. S. S. Kalter: Dr. Sigel, I don't know if there is any way of intelligently guessing at this. We find contamination is a very large cause—that is, fecal contamination. But very frequently, we will find an agent in the kidney that we can't find in the throat or stool. I think perhaps Dr. Hull or Dr. Malherbe has more data on this question.

Dr. F. Rapp: Dr. Hennessen, do you have any information concerning the susceptibility to broad-spectrum antibiotics of the agent isolated from the green monkeys that is being passed in guinea pigs? Does the use of such antibiotics modify the course of the disease seen in human patients?

Dr. W. Hennessen: Broad-spectrum antibiotics did not have any effect on the patients. And in guinea pigs, the action of chloramphenicol and terramycin was such that these guinea pigs did not develop fever. But both the treated and untreated guinea pigs died on the same day, so there was just a depression of temperature, but not depression of disease.

Dr. P. D. Cooper: Dr. Kalter gave us an impressive list of viruses that he found in monkey tissue cultures. I would like to ask him or perhaps somebody from the Division of Biologics Standards: How many of these viruses are routinely tested for in the monkey kidney vaccines that are currently in use?

Dr. Kalter: I can't answer for the Division of Biologics Standards. We are now getting into testing for different agents, but only for other interests—just now, for instance, for the presence of simian viruses in various species of primates. But as far as the tissues themselves are concerned, or vaccines, I think Dr. Hull's data show that the virus content in tissue or excreta vary tremendously from year to year. I think Dr. Malherbe has some data that he didn't present that might be more pertinent to your question. Do you want to give those data?

Dr. H. Malherbe: In recent years, we isolated 9 different groups of viruses from monkey kidney tissues. Some of these don't fall into the recognized classification and are still to be classified. They are peculiar agents, difficult to handle, but they are there.

It is extremely difficult to test a vaccine for these agents because, as I mentioned before, the antiserum applied to the vaccine as the mainstay of the test on the final vaccine, usually, I think, is prepared in large animals which can contain antibodies to the viruses one might suspect. Until one can produce an antiserum free of antibodies against the viruses that are likely to be sought, one is merely neutralizing them away with the vaccine virus one is trying to eliminate.

As far as cell production batches are concerned, we found virus recovery varies greatly from year to year. And it varies whether the monkeys are kept together or kept separate or held for long or short periods.

Over a 3-year period, we tested 1,033 monkeys, and we got 42 strains of virus. These viruses were in 6 different groups. Then, in 10½ months, we tested 615 monkey kidney batches and got 196 strains of virus. These viruses fell into 5 groups.

When the method of holding these monkeys was altered, the incidence dropped considerably. But we still recover 3 or 4 different kinds of viruses from our monkey kidneys from time to time. But there is no means of really thoroughly testing for these agents in the final vaccine.

The real mainstay is the control cultures and, I believe, the growth medium that is harvested from the cultures which are actually used in vaccine production at the point when they are to be inoculated. This test has been eliminated from the WHO recommmendations. I think it should be reinstated. It seems to me to be one of the most valuable tests.

Dr. Hennessen, have you examined your tissue cultures for virus inclusions? I mention this somewhat diffidently. We were sent sera from patients of Marburg cases to test against African arboviruses. Dr. McIntosh of our Arbovirus Unit found these sera negative against these antigens.

However there was a 6-day serum from one of the patients. This serum was put into tissue cultures of vervet monkey kidney in my research unit. The tissue cultures were apparently intact, but on the 23d day we harvested and stained the cells, and the cells contained inclusions. This material was then subinoculated into further cultures, and by the 12th day the cells again contained inclusions.

All I can say is that this is an agent which we found in the serum of one of these patients. Whether the agent is responsible for the outbreak, I don't know. But in our experience it is a new agent. It is possible to have tissue cultures which are apparently intact and which in fact do contain inclusions.

Dr. Hennessen: The answer is we had not looked for inclusion bodies up to then, but the cases which were infected from tissue cultures in our laboratories were among personnel, not working in the laboratory but in the cleaning kitchen, who had to clean material from 8-day-old tissue culture. This same tissue culture was observed for 3 weeks and we did not see anything. But we did not stain these cultures.

Dr. Sigel: Did you attempt any serological studies with patients' convalescent serum and your agent? You mentioned earlier that there is no defense mechanism detectable in these patients, but what about antibody formation?

Dr. Hennessen: The antibody formation in the patients who survived was very strong. What I mentioned was in the fatal cases. The neutralization titers in the cases in the convalescent sera were rather high. The ones done in Marburg showed titers up to 500. And the titers you find in your reports later are complement-fixation titers which seem to be very low. They don't go up to more than 1 in 64, but they were titered against a rather crude complement-fixing antigen, a virus antigen.

So the antibody formation was good in the surviving cases while there was nothing. We did some other serology on this. We did find an increase in the macroglobulins.

Dr. J. L. Melnick: Dr. Hennessen, did you look for antibodies against this agent in the monkeys shipped from Uganda and did you look in other monkey sera, that is, in normal monkey sera?

Dr. Hennessen: The investigations on monkey sera have not been completed yet, but there are unfortunately very few monkey sera left because from the monkeys which we killed, we were not able to receive blood. It took 8 hours until we could make the autopsies. We were not able to obtain a considerable amount of blood from the monkeys killed in our quarters. And there are only a very few samples of monkey blood left.

Dr. F. T. Perkins: The infectious agent from the blood of these monkeys, infecting people, has been put into guinea pigs and the guinea pig agent has been put into cell cultures. This agent is giving inclusion bodies. Dr. Gordon Smith has done this. The inference would be, therefore, that this agent is probably the same as the one Dr. Malherbe has seen in putting serum on his tissue cultures which is also giving inclusion bodies.

Dr. K. G. Brand: Dr. Malherbe, which type of inclusion bodies do you see with regard to staining characteristics and localization?

Dr. Malherbe: This is a culture of the 23d day harvest of the monkey kidney culture initially inoculated with the patient's serum.

None of our South African monkey agents produce cytoplasmic inclusions of this character without nuclear inclusions of syncytia. Of all the human agents we have screened, only parainfluenza #2 does this. But the character of the cytoplasmic inclusion is different.

These inclusions are rather deeply staining and eosinophilic. They tend to be much smaller than those of parainfluenza and of a slightly different shape. This virus is new to us. It occurred in both of the tubes that we inoculated with this patient's serum. We have never found it in South African cercopithecus monkeys. So if it does come from cercopithecus monkeys, it is coming from Central Africa. But I am somewhat surprised that it had not been found before, in view of all the monkeys from Central Africa that have been used.

Session III

Neoplastic Properties of Animal Cell Lines

Chairman: JACOB FURTH
Rapporteur: THELMA DUNN

Methods for Determining Neoplastic Properties [1]

George E. Foley,[2] *Children's Cancer Research Foundation, and Department of Pathology, Harvard Medical School, at Children's Hospital, Boston, Massachusetts 02115*

Rather than to detail the experimental methods used in attempts to characterize neoplastic cells in culture, it seems preferable to review them rather briefly, and to express a point of view—which is in large part that expressed previously (*1*, *2*)—as to the wisdom of extrapolation from such experimental systems in terms of the safety of products derived therefrom for use in man.

The precise nature of the biochemical defect(s) underlying neoplasia has not yet been delineated; thus these experimental systems at best can only provide descriptions of altered behavior in culture which may or may not indicate neoplastic origins or transformation. The primary utility of such systems relates to questions concerned with the fundamental nature of neoplasia and neoplastic transformation; but it must be remembered that evidence of altered biological behavior deriving from one experimental system is interpreted as evidence for neoplastic properties or neoplastic transformation by comparison with data deriving from still another experimental system. Thus far, these experimental systems seem to have one characteristic in common: None of them, either alone or combined, define attributes solely characteristic of, and solely peculiar to, neoplastic cells (or conversely, non-neoplastic cells) in culture. As stated recently by Federoff (*3*), there are no *in vitro* criteria which define tumorigenicity.

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[2] Holds Public Health Service research career award 1–K6–CA 22, 150 from the National Cancer Institute.

MATERIALS AND METHODS

Morphology

The time-honored criteria of the histopathologist do not necessarily distinguish between non-neoplastic and neoplastic cells in culture (table 1). Cell morphology may be profoundly affected by the composition of, or alterations in, the culture substrate. Culture-acquired morphologic alterations are well known: To cite but a single example, cells of undoubted tumor (*4*) and leukemic origins (*5*) may assume fibroblast-like morphology in monolayer cultures.

Table 1.—Some characteristics of mammalian cells in culture

	"Usual" characteristics*	
	Non-neoplastic	Neoplastic†
Morphology:		
Cellular	Not reliably distinguishing.	
Growth in monolayer	Orderly	Orderly, may be disoriented.
Chromosomes—karyotype	Normal, may be altered.	Altered, may be normal.
Growth *in vitro:*		
Maximum population density	Low	Increased.
Growth in suspension culture,	No	Yes.
soft agar.	No	Yes.
Plating efficiency	Low	Increased.
Life expectancy	Diploid, finite Heteroploid, infinite	Infinite.
Membrane changes:		
Self-adhesion	Yes	No.
Contact-inhibited	Yes	No.
Biochemical	No distinguishing markers.	Activity may be increased.
Immunological	No distinguishing markers.	

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*Of some, but not all, cell cultures. Not differential, either alone or combined.

†Similar characteristics are exhibited by cells transformed by oncogenic agents *in vitro*—not all of which are transplantable—and by nontransplantable heteroploid cell cultures derived from non-neoplastic tissue.

Disoriented or random growth patterns and a tendency to grow as dense, highly compacted colonies, as opposed to the usually more orderly orientation of cells in monolayer cultures (table 1), are characteristic of many virus- or carcinogen-transformed cells. But all cells exhibiting such altered growth patterns do not produce tumors in experimental hosts (*6*).

Chromosomes

As yet, no acceptable evidence relates specific chromosomal aberrations causally to neoplasia (*7*), though some evidence suggests that neoplastic transformation involves alterations (*8*, *9*) in the nucleolar-heterochromatin system (*10*). With respect to cells in culture, there is no reasonable basis for equating the diploid state with "normal"; also there is no reasonable basis for equating the heteroploid state with neoplastic properties (table 1), as judged by the biological behavior of such cells in experimental hosts. Some diploid or near-diploid cells are transplantable in experimental hosts (*1*, *11–13*); and some evidence exists that, in virus transformation *in vitro*, cells may acquire neoplastic properties before any detectable change in karyotype (*14*). Furthermore, heteroploid cell lines derived from non-neoplastic tissue fail to produce tumors as judged by homotransplantation or heterotransplantation studies in experimental hosts (*15*).

Karyotypic aberrations usually accompany neoplastic transformation, but they may occur independently of such transformation in culture. In other instances they appear to be secondary to the primary occurrence inducing neoplastic transformation, in that they develop only with the progression of tumor toward complete autonomy (*14*). For example, not all cells karyotypically transformed as a result of exposure to virus *in vitro* can produce tumor in experimental hosts. There is an interesting parallel to be drawn here: The finite lifespan of many non-neoplastic cells which remain diploid or near-diploid *in vitro* (*16*) contrasts with the infinite lifespan of non-neoplastic cells which have become heterploid *in vitro* for reasons not yet clearly understood (perhaps by adaptation to environmental changes, or as a result of exposure to commensal virus), but which do not produce tumors in experimental hosts (*15*).

On the other hand, the argument is untenable that chromosomal alterations are unrelated to neoplasia or to the acquisition of neoplastic properties. Within the context of current DNA dogma, the biochemical defect(s) implicit in the biological definition of neoplasia should reside either in the chromosomal nucleic acids or in the transcription mechanisms regulating biosynthesis. Present evidence clearly indicates that such subtle alteration(s) may well be at the molecular level, and that more sophisticated hypotheses, instrumentation, and experimental approaches will be required for its (their) detection and delineation (*17*, *18*).

Plating Efficiency

Most, but by no means all, neoplastic cells in culture are heteroploid. Unlike diploid cells, heteroploid cells generally are not contact-inhibited (*6*). Hence, the population density which can be attained in culture usually is higher for heteroploid than for diploid cells (table 1). Also, most diploid cells do not grow in suspension culture, whereas heteroploid cells may be adapted to such cultures. These differences in growth characteristics are reflected in the higher plating efficiency of heteroploid cells, which may be related to the ability of some heteroploid neoplastic cells to form colonies in soft-agar plates (*19*). Such differences appear to be more closely related to the state of ploidy rather than to the neoplastic properties of a particular cell line; and none of these properties are characteristic of, and peculiar to, either all diploid or all heteroploid cell lines in culture.

For example (table 2), the CCRF–CEM cells, isolated from the peripheral blood buffy coat of a pediatric patient with acute lymphoblastic leukemia (*20*), are of classical lymphoblastic morphology in culture (*20*, *21*). They are near-diploid (*22*), but have been serially transplanted in newborn Syrian hamsters (*Mesocricetus auratus*), where they present a striking recapitulation of the disease in man (*12*). However, these cells will not grow in the usual monolayer culture or in soft agar plates, and indeed they grow properly only in suspension cultures in which population densities of 1–5 × 10^6 cell/ml are routinely maintained in volumes ranging from 1–20 liters or more of substrate (*18*). As judged by several of the usual "cultural characteristics," these cells would be classified as non-neoplastic, although they implant, invade, metastasize, and produce leukemia in newborn Syrian hamsters.

TABLE 2.—Some cultural characteristics of human leukemic lymphoblasts (CCRF–CEM cells): Comparison with some nontumorigenic cell lines

	Cell lines of non-neoplastic origins		CCRF–CEM cells
	Diploid	Heteroploid	
Chromosomes	Normal	Altered	Normal.
Growth *in vitro:*			
Suspension culture	No	Yes	Yes.
Soft agar	No	Yes	No.
Maximum population density	Low	Increased	Unusually high.
Plating efficiency	Low	Increased	Unusually low.
Life expectancy	Finite	Infinite	Infinite.
Tumorigenicity in Syrian hamsters	No	No	Yes.

Membrane Changes

As Weiss and Mayhew (*23*) pointed out in a recent review of the cell periphery, although there is an abundance of experimental data on membrane changes, its interpretation is equivocal. Many surface properties thought to distinguish between non-neoplastic and neoplastic cells have been found to be quantitative rather than qualitative differences.

More than 20 years ago, Coman (*24*, *25*) described the decreased intercellular adhesiveness of neoplastic cells (table 1). The effects of calcium and magnesium ions and of nucleoside phosphates (*26*) on adhesiveness and motility are well known. In culture, the same cells (either normal or neoplastic) do not exhibit the same degrees of adhesiveness or of motility either under different experimental conditions or in different substrates; there are neoplastic cells which characteristically form large aggregates (*27*, *28*) even in suspension culture—*e.g.*, the lymphocytic cells derived from Burkitt's tumor (*29–31*)—suggesting that all neoplastic cells have not lost the property of self-adhesion.

Abercrombie and Heaysman (*32*) described the loss of cell motility (contact inhibition) in crowded cultures of fibroblasts, and the loss of the property of contact inhibition (table 1) was thought to be characteristic of neoplastic cells (*33–36*). However, inhibition of neoplastic murine cells by contact with normal cells has been reported (*37*), as has contact inhibition of virus-transformed hamster cells by normal hamster or mouse cells (*38*, *39*). In other instances, the molecular occurrences resulting in cessation of growth (*40*) in contact-inhibited cells appear to be induced by contact with the surfaces of the same or other kinds of non-neoplastic cells, whereas cells transformed by oncogenic agents are contact-inhibited only by contact with the surfaces of cells transformed by a different oncogenic agent (*41*). Other evidence suggests that contact inhibition may be related to, if not dependent on, type-specific phenomena (*6*)—reminiscent of the formation of homotypic aggregates in mixed cell cultures (*42*). Recent studies (*43*) suggest that cell motility is oriented toward the surface offering greater adhesion. Thus, loss of cell motility may well be due to lack of adhesion differentials between the cell and the surfaces available for attachment; and a unifying interrelationship between cell adhesion and cell motility has been proposed (*44*).

Although changes in adhesiveness and loss of contact inhibition may sometimes be associated with neoplastic cells or the acquisition of neoplastic properties, these phenomena do not appear to be specific and as yet are too poorly understood to be useful as differential criteria.

Biochemical Activity

As Potter (*45*) stated so succinctly: "The biochemistry of cancer is a subject about which little is known and much has been written." As yet no biochemical marker or combination of biochemical markers is known which will distinguish between normal and neoplastic cells. Chromosomal aberrations may be accompanied by alterations in the biochemical activity of cells in culture—*e.g.*, increased alkaline phosphatase or cystathioninase activity (*6*)—but such alterations appear to be more closely related to karyotypic aberrations than to neoplastic behavior (table 1).

Although induced viral or chemical carcinogenesis in experimental systems frequently, but not invariably, results in new specific antigens at, or on, the cell surface, there is as yet little evidence for the presence of similar antigens in human neoplastic cells; indeed, even in experimental carcinogenesis, the precise relationship of such new antigens *per se* to neoplastic transformation is not yet understood, since similar antigens have been reported to appear in the noninvolved tissues of tumor-bearing hosts (*46*). At the present time, reliable antigenic distinction cannot be made between normal and neoplastic cells.

Transplantation

By definition, the progressive growth of transplanted cells, like that of transplanted biopsy specimens, should distinguish non-neoplastic from neoplastic cells, though failure of the implanted cells or tissue to grow does not necessarily establish that the specimen was "non-neoplastic." These classical criteria, despite their shortcomings, may be applied to the evaluation of nonhuman cell cultures by means of transplantation studies in isogeneic or syngeneic hosts. But with human cell cultures, evaluation by such means is further complicated by the necessity of using xenogeneic hosts.

Xenogeneic transplantation studies using cortisone- or X-irradiation-conditioned rats, mice, or Syrian hamsters are well known, as are Greene's studies on the differentiation of non-neoplastic and neoplastic tissue by transplantation to the anterior chamber of the guinea pig [*cf.* (*15*)]. The studies of Handler *et al.* [*cf.* (*15*)] clearly indicated that a variety of human neoplasms may be transplanted to the Syrian hamster, and that the Syrian hamster is endowed with a unique capacity to distinguish between neoplastic and non-neoplastic tissue; the former is accepted, whereas the latter, with few exceptions, is promptly rejected.

Some time ago, for reasons (*5, 47*) not germane to the present discussion, we were interested in distinguishing between cell lines derived from non-neoplastic and neoplastic tissue, if indeed such distinction were possible. Lacking any other suitable marker, we compared the biological behavior of cell cultures when transplanted into the cheek pouch of the Syrian hamster. This host was selected for such studies for two reasons: 1) The background data were already available on the acceptance (with a minimum of "conditioning" with cortisone acetate) of heterologously transplanted neoplastic tissue taken directly from the patient and rejection of similarly derived normal or benign tissue taken directly from the patient; and 2) these studies were concerned primarily with cultures of human cells, although included in this series of experiments (*5*, *15*, *48–51*) were cell cultures derived from non-neoplastic, neoplastic, adult, and embryonal murine tissue as well. All cell lines were established *in vitro* and all were heteroploid.

The biological behavior of these cell lines in the hamster cheek pouch correlated remarkably well (table 3) with the nature of the tissue of origin with few exceptions: Unlike the frankly invasive, transplantable tumors produced in the cheek pouch by those of neoplastic origins, the nodules resulting from the implantation of even larger doses of cells of non-neoplastic origins were noninvasive, elicited intense inflammatory responses, regressed promptly, and were not serially transplantable (*15*, *50*). Cell lines derived from embryonal tissue proved an exception however (table 3): A number of such lines were indistinguishable from those derived from frankly neoplastic tissue with respect to their ability to grow progressively in the hamster cheek pouch (*15*).

Cytochemical studies with these cell lines (*52*), using biophysical instrumentation developed for cytochemical population analyses, indicated that those cells derived from neoplastic tissue have a greater variability in the amount of cytoplasmic protein and cytoplasmic RNA per cell, which has been described in earlier studies with populations of cells derived directly from *in vivo* sources (*53–56*). It is interesting that these cytochemical attributes of populations of neoplastic cells *in vivo* are retained *in vitro* and that they correlate with the xenogeneic transplantability of such cell lines in the cheek pouch of the Syrian hamster (table 3).

TABLE 3.—Xenogeneic transplantation of mammalian cell lines: Comparison with biopsy specimens

Tissue of origin	Tumorigenicity in Syrian hamsters			
	Non-neoplastic		Neoplastic	
	Cell cultures	Biopsy specimens	Cell cultures	Biopsy specimens*
Adult, diploid or pseudodiploid	0	0	+	+
Adult, heteroploid	0	?	+	+
Cytochemical abnormalities	0	0	+ †	+ †
Embryonal, diploid or pseudodiploid	0	?		
Embryonal, heteroploid	0 or +	?		

*Certain varieties of human tumors have not yet been transplanted to the Syrian hamster, and apparently non-neoplastic cells (stromal?) are occasionally derived in cultures [*cf.* (*15*)].

†Increased variability in cytoplasmic protein and cytoplasmic RNA content per cell in these cell populations as compared to nontransplantable cell populations derived from non-neoplastic tissue.

DISCUSSION

Thus, a series of phenotypic changes has been observed in neoplastic cells and in non-neoplastic cells as a result of transformation by virus or other carcinogenic agents. None of these changes (table 1) are characteristic of, or peculiar to, all neoplastic cells, although as Eagle and Levine (*6*) pointed out, these changes frequently show a high degree of correlation and may well be causally interrelated. These changes have not yet revealed the nature of the biochemical defect(s) intrinsic in the biological definition of neoplastic behavior; however, the fact that these changes may not occur in cells which exhibit neoplastic behavior as defined in experimental transplantation systems, whereas they may occur in cells which do not exhibit such behavior in the same experimental transplantation systems, suggests that highly specific alterations at the molecular level are essential for the expression of the biological characteristics used to define neoplastic as opposed to other kinds of cellular transformation.

It is tempting to view the production of tumor in an experimental host as an expression of genotypic rather than phenotypic change. The cytochemical variability characteristic of populations of neoplastic cells might be interpreted in support of such a viewpoint (table 3), in that the magnitude of the variability of cytoplasmic RNA content per cell is such that it must represent quantitative variations in the ribosomal RNA content of neoplastic cells—which in itself implies defects or disturbances in the nucleolus-nucleolus-associated chromatin biosynthetic system (*8*). Indeed, some evidence suggests that the nucleolar metabolism of tumor cells may differ from that of other cells (*57*).

The behavior of cultured cells in transplantation systems, despite its obvious shortcomings, is perhaps the best available means of distinguishing between non-neoplastic and neoplastic cells; but even presuming precise differentiation of cells *e.g.*, of murine origins, even in an isogeneic transplantation system, such distinction does not necessarily assess the nononcogenic (or conversely, the oncogenic) potential of derivatives of such cell systems for man. In xenogeneic transplantation studies in the Syrian hamster, particularly those done with cell lines of human origins, there is no direct evidence that the biological behavior of a given cell line necessarily reflects the potential behavior of that cell line or its products in man. The correlations between the source of the cell line and its biological behavior in the hamster cheek pouch are remarkably good (*15*), but extrapolation from such data can be made only by inferential argument from the observation that biopsy specimens obtained directly from man behave in a similar manner—a tenuous argument which obviously may or may not be tenable (*1*, *2*).

The results of transplantation studies might be interpreted to indicate that the production of tumor is acceptable inferential evidence for neoplastic or potentially neoplastic properties in man, but the obverse is untenable—that failure to produce tumor in an experimental host is acceptable evidence for the lack of neoplastic or potentially neoplastic properties. Indeed, with human cell lines, the point of view has been expressed that their tumorigenicity could be evaluated validly only by implantation in the original donor; and even the validity of this kind of assay could be questioned (*6*).

In addition to the risks inherent in inferential arguments based on such transplantation systems, there is little question that neoplastic transformation (as defined in transplantation systems) does indeed occur, particularly in cultured cells of nonhuman origin (*58*, *59*), due in many instances to exposure to commensal virus. Further, it would not be surprising if indeed neoplastic transformation occurs without the mediation of virus. Neoplastic transformation could conceivably result from the exchange of genetic information between cells by hybridization or other means, accidents in cell division, functional incorporation of fragments of exogenous nucleic acids—any agency which effectively disrupts the normal regulatory mechanisms of the cell and results in alterations in protein synthesis or its regulation—the synthesis of new antigens, or the deletion of specific proteins (*60*).

Since the means of assessing the potential hazard for man of these phenomena, or that of undetected preneoplastic change as discussed elsewhere (*2*), are not immediately at hand, it is unreasonable to expect that the characterization of neoplastic behavior in experimental systems will effectively resolve the fundamental issue as to the safety of products derived from such systems when used in man. A more definitive solution appears to be the development of purified vaccines freed of extraneous products deriving from the cell system in which they have been produced.

SUMMARY

The methods employed in attempts to characterize neoplastic cells in experimental systems are reviewed briefly. Since the precise nature of the biochemical defect(s) underlying neoplastic behavior has not yet been delineated, these experimental systems only provide descriptions of phenotypic changes which may or may not accompany, and may or may not be indicative of, the neoplastic origins of the cells or neoplastic transformation. None of them, either alone or combined, define attributes which are solely those of neoplastic (or non-neoplastic) cells in culture.

The behavior of the cells in experimental transplantation systems, despite obvious shortcomings, remains the best available means of defining the tumorigenicity of cultured cells. Tumorigenicity in experimental hosts might be interpreted as acceptable inferential evidence for neoplastic potential in man; but the obverse—lack of tumorigenicity in experimental hosts—cannot be interpreted to indicate a similar lack of neoplastic potential for the products derived from such a cell system for use in man. The fundamental issue might be best resolved by the development of purified virus vaccines.

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Methods for Determining Neoplastic Properties—Discussion

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Dr. FOLEY's exposition of the problems involved in identifying neoplastic properties has been so thorough that it leaves little to be added. My comments will serve only to amplify his views and will take note of two additional aspects of the problem of identifying undesirable cells.

To do this, I draw your attention to similarities between the concepts underlying the use of the terms "pathogenic organism" and "neoplastic cells." "Virulence" and "malignancy" are two additional roughly comparable terms often used to express quantitative aspects of pathogenicity of microorganisms or neoplastic properties of cells, respectively. We have been advised that we will be on more acceptable ground in this gathering if we speak of oncogenic cells and oncogenic characteristics, rather than of neoplastic cells and malignant characteristics. So I will follow that recommendation.

Terminology aside, we cannot escape the point implicit in Dr. Foley's presentation—namely, that virulence (or oncogenicity) of animal cells, like virulence of microorganisms, is something that must be expressed in relation to size of dose, site of inoculation, species, strain, and genetic constitution of the recipients, immunologic status of the recipients, and many other factors such as endocrine status, nutritional status, age, temperature, latent period, and so on. Virulence of cells, like virulence of microorganisms, is something that can be assayed only in relation to some specific biologic context. At the moment, one cannot assay either virulence or oncogenicity as one can assay, for example, the quantity of iron in a sample of ore. One must always say: "Virulent under what conditions?" or "Oncogenic under what conditions?"

What we do look for are phenotypic traits of cells *in vitro* that will serve as *indicators* of their oncogenicity under more or less particular conditions *in vivo*. This approach compares with the analysis of bacterial cultures for production of toxins, certain enzymes (*e.g.*, collagenases, coagulases, hemolysins), capsular polysaccharides, or specific antigens.

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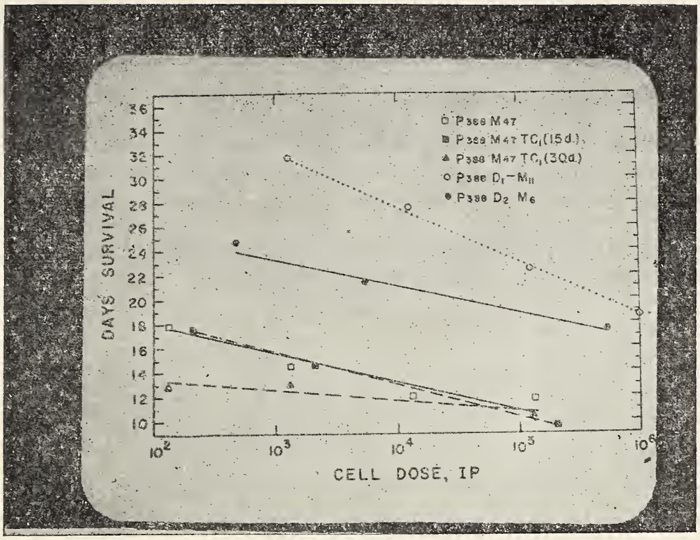
Dr. Foley has already referred to membrane characteristics, contact effects, plating efficiency, antigens, transplantation results, and biochemical activities as measures of neoplastic character in animal cells. To the biochemical activities of importance should be added those which relate to the ability of cells to digest collagen, basement membranes, and other connective tissue components that serve as natural barriers to the spread of cell populations. Little enough work has been done in this field, but the early investigations of Santesson (*1*), and the later ones of Leighton (*2, 3*), and of Gey (*4*) have shown that differences exist among cells regarding their abilities to digest and/or penetrate such things as coagulated egg albumen, gelatin, plasma clot, collagen, and ground substance of umbilical cord. Unfortunately, here as with other tests for neoplastic capabilities, no correlations have yet been established that permit reliable prediction of behavior *in vivo*.

Secondly, it ought to be recognized that cells can *lose* as well as gain oncogenic capabilities while maintained in continuous culture. Perhaps the first strong indication of this was reported by DeBruyn (*5*), who found that cells from a murine lymphoma lost their ability to produce tumors after being cultured free of normal fibroblasts. A year later, Earle *et al.* (*6*) reported that several mouse cell lines, which had undergone "spontaneous" neoplastic conversion in culture, were declining in oncogenicity. In 1958 we described a gradual decrease of tumor-producing ability by a reticulum cell sarcoma of mice (*7*), and also in 1957 by a lymphoid neoplasm of mice (*8*). Text-figure 1 presents data showing the prolonged survival of mice inoculated with Lymphoma P388 cells from ascites tumors derived from cultivated P388, as compared with data on survival of mice inoculated with equivalent doses from the parent tumor.

There have been several repetitions of this kind of observation by other investigators. Hsu and Klatt (*9*) have shown the loss of tumor-producing ability by the Novikoff rat ascites hepatoma after prolonged cell culture, and Cailleau and Costa (*10*) reported comparable findings for the Ehrlich ascites tumor in culture. Guerin and Kitchen (*11*) made similar observations regarding the 6C3HED lymphoma after adaptation to culture. Such events are reminiscent of the loss of virulence that sometimes occurs in pathogenic bacteria after prolonged culture in laboratory media.

The reasons for loss of oncogenic properties by cells in culture may be various. For example, they may develop new antigens, or they may lose synthetic mechanisms favorable to rapid growth in the natural host. The pathways to nononcogenicity are perhaps not of great importance to the objectives of this meeting. However, if one starts with the premise that cells of neoplastic derivation are undesirable for viral vaccine production, then knowledge of the phenomenon of attenuation of oncogenicity requires that one take note of a cell line's past activities, as well as its present performance. This is a small point that rarely presents practical difficulties.



TEXT-FIGURE 1.—Dose-survival time relationships for BALB/c × DBA/2 mice inoculated with 5 cell sublines derived from mouse Lymphoma P388. Each *point* represents the mean survival time for 8 recipients. P388 M47 represents ascites tumor cells from 47th serial passage in mice, without any time in culture. P388 M47TC$_1$ (15d.) represents ascites tumor cells from 47th passage in mice preceded by one 15-day period in culture. P388 M47TC$_1$ (30d.) represents the same, but preceded by a 30-day period in culture. P388D$_1$–M$_{11}$ represents cells from the 11th serial passage in mice of P388 cells adapted to continuous cultivation. P388D$_2$–M$_6$ represents cells from 6th serial passage in mice of P388 cells adapted to continuous cultivation, but from a separate isolation.

Note lengthened survival times for mice receiving ascites tumor cells derived from culture-adapted cells (*2 upper lines*).

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Methods for Determining Neoplastic Properties—Discussion

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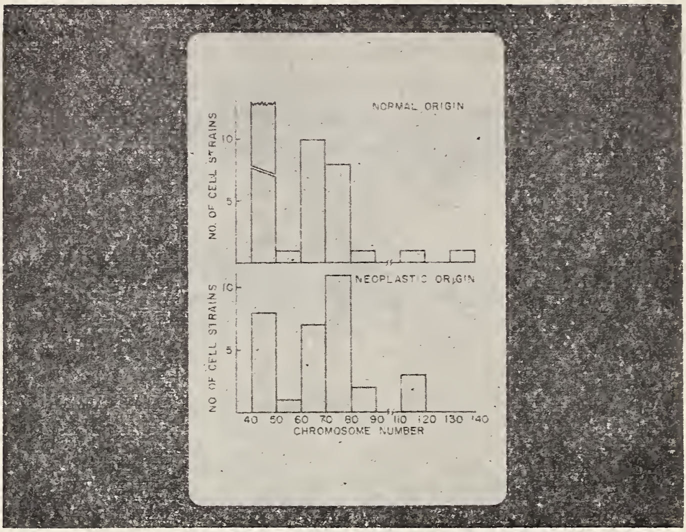
In the very short time allotted, it is feasible to touch upon only a few of the features of chromosomes in human leukemia and cancer patients and the possible relationship and application of these findings to the definition of neoplastic properties of cells grown *in vitro*. No consistent or specific karyotypic abnormality has been encountered in human cancers, whether primary or metastatic, and no two tumors have been found to have identical karyotypes (*1*, *2*). Furthermore, in our laboratory we have not found a single diploid human cancer. This observation contrasts with our findings in human acute leukemia, in which about 50% of the cases are accompanied by diploidy (*3*).

The aneuploid acute leukemias, except for a small group associated with group-C trisomy, are characterized by an impressive variability in chromosome number and constitution, with no two leukemias being identical. The only consistent and seemingly specific karyotypic abnormality in human neoplasia is the Ph^1 chromosome in chronic myelocytic leukemia (CML). We contend that the chromosomal changes in human cancer and acute leukemia patients are secondary to the neoplastic state and induced by it—except possibly the Ph^1 chromosome in CML which may, in fact, be the direct cause of this disease (*4*). Thus, we envision the neoplastic state to be the result of abnormal function of the chromosomal DNA and not necessarily accompanied by visible chromosomal changes. When the latter occur they are of extreme diversity and may possibly reflect the extreme variation in the human genomes rather than the cause of the neoplasm.

With one possible exception, all cell lines derived from human cancer have been aneuploid (text-fig. 1). Furthermore, most established cell lines have a modal chromosome number between 60 and 80 and often exhibit abnormal chromosomes, so-called markers (*5*). A substantial number of cancer cell lines had a modal number in the diploid range (40–50 chromosomes).



Text-figure 1.—Distribution of modal chromosome numbers in established cultures originating from normal and neoplastic cells. Text-figure is based on data secured from the literature and from our laboratory. Among the cell lines originating from neoplastic cells, none of those having a chromosome number between 40 and 50 had a normal karyotype.

Similar are the findings obtained with cells of non-neoplastic origin. Even though it has been possible to maintain diploidy in human cell lines for amazingly long periods (5 years or more) the data in tables 1 and 2 show some of the perplexing problems encountered. In the first case (table 1), a blood sample obtained from a patient was divided into 13 cultures and grown under approximately identical conditions. Only 8 lines maintained diploidy, whereas the remaining 5 became frankly abnormal, with one line developing a mode of 48 chromosomes. In the second case (table 2) 2 of 5 cultures developed karyotypic abnormalities. None of the cell lines has been shown to have neoplastic properties, either by homotransplantation or heterotransplantation.

It is possible for some diploid lines to have neoplastic characteristics, as it is possible to have diploidy in human acute leukemia, if at least the physiology of the DNA has been modified to express itself neoplastically. But, most human cell lines with a predominantly diploid number of chromosomes appear not to have neoplastic features evident with presently available tests. The very same shortcomings of the testing systems may be responsible for the observation that very heteroploid cell lines are often neoplastic and only an occasional one fails to demonstrate neoplastic behavior. This may also account for the rarity of finding a human neoplastic line that is in the near-diploid range. It is hoped that more sensitive and reliable systems will become available to ascertain the neoplastic characteristics of cell lines than those presently available.

TABLE 1.—Chromosome numbers and outstanding karyotypic findings in a diploid human cell line (P) (100 metaphases examined for each sample)

RPMI cell line No.	Modal chromosome No. (%)	< Modal No. (%)	> Modal No. (%)	Percent polyploidy	Abnormality
5298	46 (93)	44–45 (6)	52 (1)	2.6	None
5763	46 (86)	44–45 (13)	47 (1)	6.8	None
5759	46 (92)	42–45 (7)	47 (1)	1.4	None
5477	46 (91)	44–45 (8)	54 (1)	3.2	None
5951	46 (87)	42–45 (13)		2.2	None
4226	46 (88)	42–45 (12)		0.5	None
4216	46 (73)	42–45 (22)	48–52 (5)	4.4	None
5956	46 (87)	43–45 (11)	52–66 (2)	3.7	None
4265	46 (91)	44–45 (8)	49 (1)	1.0	Iso-D?
7865	46 (84)	43–45 (15)	47 (1)	1.6	Iso-D/translocation?
5301	46 (85)	43–45 (15)		2.5	Long D/normal
4246	46 (84)	42–45 (15)	47 (1)	0.4	Long E18/normal
5166	48 (38)	44–47 (59)	49–50 (3)	3.6	Extra D and G

TABLE 2.—Chromosome numbers and outstanding karyotypic findings in a diploid human cell line (C) (100 metaphases examined for each sample)

RPMI cell line No.	Modal chromosome No. (%)	< Modal No. (%)	> Modal No. (%)	Percent polyploidy	Abnormality
8205	46 (86)	44–45 (14)		1.6	None
8195	46 (84)	44–45 (16)		1.5	None
0295	46 (92)	44–45 (8)		1.1	None
8265	46 (88)	44–45 (9)	47 (3)	2.4	Three A3 autosomes
8235	46 (87)	41–45 (12)	48 (1)	1.2	Long D/normal

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GENERAL DISCUSSION

Dr. H. W. Toolan: I enjoyed very much Dr. Foley's presentation. I do feel, however, that exception should be taken to his table 3 which shows that there is considerable correlation between the origin of the cells—that is to say, whether they are normal, embryonic, or malignant—and their growth in the hamster pouch. Many normal cells, particularly those of skin and thyroid, grow quite well for one generation in the hamster pouch. Embryonic cells, except liver, proliferate especially well.

Dr. G. E. Foley: I agree with Dr. Toolan. As I recollect in my manuscript, I said some cells of embryonal origin in our hands behaved exactly the same way they behaved in Dr. Toolan's hands—like cells derived from neoplastic tissue.

Dr. J. Furth: Also they are not invasive, the growth remaining in the pouch.

Dr. Foley: Yes. Histologically, they have the same characteristics that one sees in the pouch implanted with a KB cell, for example. They have the same growth rate, high mitotic index, and lack of host response, completely indistinguishable, one from the other.

Dr. M. M. Sigel: Despite the obvious limitations, the transplantation parameter is still the only one that perhaps can tell us whether a cell has been converted to malignancy. And we must recognize the limitations.

I would like to bring up one point that perhaps hasn't been thought of; that is the number of cells transplanted. One tends to think that the more cells one transplants, the better take one will obtain. That is not necessarily so. We have exidence that, by exceeding certain cell numbers, one may rapidly immunize the host and, thereby, encourage rejection.

Dr. L. Hayflick: Dr. Toolan, will you give us some more information on the source or kind of embryonal cells that you found grew in the hamster cheek pouch? Are they, in fact, minces from embryonal tissue or primary cultures? In our hands and in the hands of those in several dozen other laboratories who have inoculated passaged cell populations derived from normal human embryonic tissue under similar circumstances, this type of growth does not occur.

Dr. Toolan: Most of our work, of course, has been done with cell minces. However, we have also trypsinized human embryonic cells and had excellent growth from them. These are fresh tissues derived from the embryo without intervening cell culture. We have had less experience transplanting embryonic cells from culture; in general they have not done as well as the primary materials. However, we have obtained some growth from them.

Dr. Furth: I would like to comment on what Dr. Sigel and Dr. Dawe said. It is an interesting phenomenon that neoplastic cells can turn non-neoplastic in the course of further transplantations. This may be more apparent than real. Successive transplantation may lead to gain in immunologic capacity, the cells remaining neoplastic but becoming transplantable only in conditioned hosts. The reverse, maturation *in vitro*, described with childhood and neurogenic tumors, has also been reported. The latter is probably related to a disturbance in differentation.

Dr. C. J. Dawe: I can't say too much because we don't know too much about it. But I didn't show here that one of our cell lines eventually went on and became completely incapable of producing tumors when transplanted intraperitoneally.

Dr. Furth: With large numbers of cells in a normal host?

Dr. Dawe: Yes, large numbers of cells in a normal host of the same strain. However, we were again able, by using some special techniques, to get them to grow *in vivo*. This was done first by X-raying the recipients and secondly by injecting the cells intravenously into newborn mice. In both cases, although tumors eventually came up from the inoculum, a very long latent period—up to 16 months—was required. That amounts to more than half the lifetime of most DBA/2 mice.

So, if one could attenuate this neoplasm just a little bit more, the mouse might live its full span before the transplanted tumor could grow to detectable size.

Dr. Furth: It is worth a venture. It is critical that the radiation resistance facet to that doesn't help.

Dr. Dawe: Presumably by putting it into the newborn mice, we were taking advantage of a tolerant period. Yet, the cells grew only very slowly.

Dr. Furth: Tumor cells can survive, but may not grow until changed host factors favor their replication, as noted with some endocrine-related tumors. Similarly, changes in immunocompetence can alter tumor growth in either direction.

Dr. Dawe: Also we tried to immunize some of these mice with attenuated cells, but were unable to do so because the cells hadn't completely lost their ability to produce tumors.

Dr. K. Sanford: Does the normal embryo tissue that can grow invasively in the hamster cheek pounch continue to grow upon transplantation or do the cells come to a halt in their growth and invasiveness?

I think we have to be very careful about talking about loss of tumorigenicity if there is any implication of a decrease in the neoplastic property. In all the cell lines we have examined where there has been a decrease in tumorigenicity, we have been able to show that the cells had become antigenic to the host.

Dr. Furth: As Dr. Dawe pointed out, tumorigenicity is a relative matter between certain cells and certain hosts.

Dr. Toolan: I think there has been some misconception of what I said. At no time did I indicate that embryonic tissues had become invasive. They definitely do not. They can survive in the same hamster, properly conditioned, for as long as 6 months or more, grow progressively, and look splendid. However, they fail to transplant well. If there is any particular criterion that can be applied to distinguish normal embryonic or malignant cells in the hamster cheek pouch, I should say that it would be whether or not the tissue could be transplanted to a second generation.

Dr. G. Barski: It is fairly well established that oncogenic properties of cell lines can increase during culture *in vitro*. However, I would like to share with you an experience we had with a cell line derived from the lung tissue of C57BL mice in our laboratory in which the reverse occurred. We obtained this result by certain manipulations rendering this line nononcogenic in terms of production of tumors in the isogenic animal. We did it by infection with the Rauscher leukemia virus.

Now, we have in our laboratory the original P–4 line which is tumorigenic and paralleling it the same line which is carrying the Rauscher virus and no longer can produce tumors by virtue of its increased antigenicity. The cells of the two lines are proliferating with the same speed *in vitro*. So this is a possible explanation of what can occur naturally—the acquisition of a new antigen induced by a virus infection of a virtually invasive cell population.

Dr. R. E. Luginbuhl: Dr. Dawe, are those animals surviving injections of attenuated cells immune to injections of cells that are not attenuated?

Dr. Dawe: No, they are not. We tried to immunize and had some animals that survived the attenuated cell inocula. But when challenged with the parent cell line, they developed tumors and died.

Dr. G. Yerganian: One aspect which should be considered at this time, in an effort to link up with the earlier session, is concerned with tumor progression and karyotypic alterations. When one clones a neoplastic cell population before implanting cultured cells into animals, one generally observes that *a*) diploid sublines form tumors much more slowly than aneuploids; *b*) diploidy may be retained for many passages only in a limited number of sublines; while *c*) most clonal sublines shift toward aneuploidy within 3 passages. Consequently, when one tends to transplant and maintain only rapidly enlarging tumors without cloning previously, aneuploidy will predominate immediately. As a result, tumor progression is associated with aneuploidization. However, by cloning first and then inoculating selected sublines into animals, the balanced diploid karyotype of neoplastic cells can be maintained both *in vitro* and *in vivo.*

Dr. Furth: Yes, analysis of this situation calls for isolation of clones of the altered cells and their comparative assay for various features of antigenicity, karyology, etc.

Virus-Associated Gain and Loss of Proliferative and Neoplastic Properties of Normal and Virus-Transformed Diploid Cell Lines[1]

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A relatively brief period has passed since the publication of the Proceedings of the 1963 Symposium held in Optaji, Yugoslavia, on the "Characterization and Uses of Human Diploid Cell Strains" (*1*). On that occasion, a number of participants—namely, Foley and Handler, Green and Todaro, Habel, and Moorhead—reviewed the experimental approaches and data pertinent to this recurrent topic: "Evidence for neoplastic properties of primary cultures and diploid cell lines." In lieu of reassembling the many negative reports on this subject, my colleagues and I will concern ourselves at this time with properties of certain "exceptional" diploid cultures that have appeared on the scene since 1963.

One of our discussants, Dr. Defendi, will describe his attempts to reveal potential neoplastic alterations that may occur *in utero* by the implantation of embryonal minces into homologous hosts. In turn, Dr. Todaro will discuss the increased sensitivity of human diploid fibroblast-like derivatives to SV40 transformation when cells are derived from individuals having known mutant genes and chromosomal imbalances. Aside from the built-in nutritional safeguards of the perfusion-cell culturing technique developed by our last discussant, Dr. Kruse, the multilayering tendency of perfused normal diploid cells may, indeed, be of practical importance for large-scale virus vaccine production.

The cell types I will discuss are derived from dwarf species of hamsters, namely, the Chinese hamster (*Cricetulus griseus*) and Armenian hamster (*Cricetulus migratorius*), each with a diploid chromosome number of 22. The most outstanding feature of the various types of cultures isolated from these two species is the relative stability of the diploid complement during the many transfer generations *in vitro* and in some instances, *in vivo*. When the karyotypes deviate in number and form, the near-diploid range, including pseudodiploidy, is generally the extent of variation; polyploids and myxoploids are rarely noted, except in occasional clonal sublines. The number of passages that diploidy is retained among normal derivatives of the Chinese hamster is in some ways indicative of one's ability in handling cell cultures. The relatively high plating efficiency (10–20%) of normal, neoplastic, and virus-transformed diploid cells encourages clonal isolations, which in turn add measurably to safeguards against early aneuploidization by the more unstable cell types observed in mixed normal embryonal populations even after many transfer generations. Diploidy can be retained from 20–125 transfer generations. This provides ample time to establish baselines for a number of phenotypic parameters which are either obscured or accentuated when aneuploidy eventually dominates all uncloned cultures.

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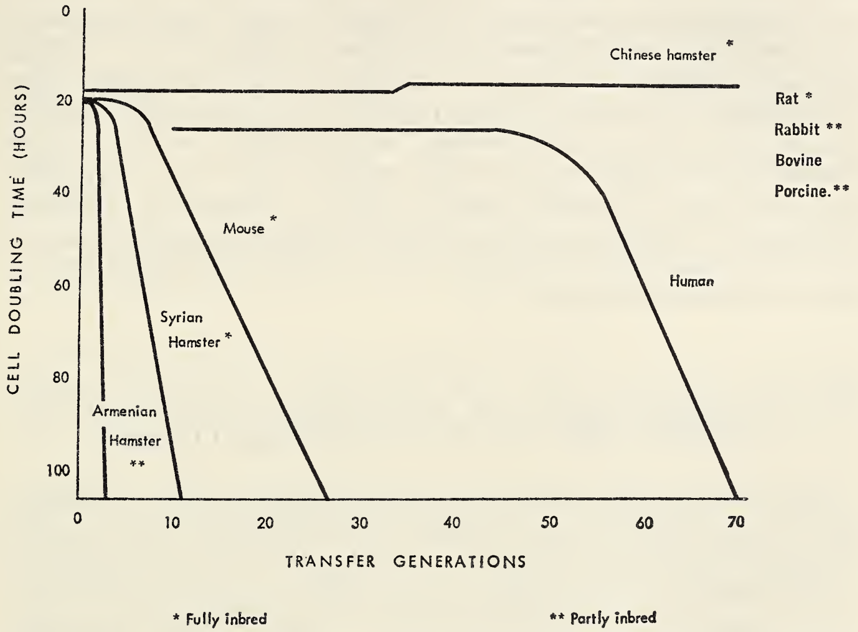
Descriptions of the general properties of normal, neoplastic, and virus-transformed cell lines will be briefly mentioned, in order to concentrate on "exceptional" cell lines from each of these categories whose properties can be related to some innocuous viral episode. These rare instances include: *a*) acquisition of neoplastic properties in an embryonal culture following the appearance of a simian virus (SV)-like agent, *b*) potentiation of *in vitro* proliferation of a "spontaneous" tumor from which a "hamster-specific," adeno-like virus was isolated, *c*) establishment of a permanent epithelial (renal?) cell line after exposure to live parainfluenza type-3 virus, and *d*) loss of neoplasitc properties and neoantigen from adenovirus 12-induced tumor derivatives after reverting *in vitro* from an epithelial- to a fibroblast-like morphology.

GROWTH RATES AND KARYOLOGICAL TRENDS DURING EARLY PHASES OF *IN VITRO* ADAPTATION

A cell line is referred to as being diploid when 75% or more of the metaphases exhibit the karyotype of normal cells described for the species. For the sake of simplification, the average cell population of a given primary culture derived from many species may be considered to include initially at least 10% mixed aneuploids. These numerical and/or structural variants may either stem from the trypsinized explant, or more likely, arise from early nondisjunctions during the early phases of *in vitro* adaptation.

The degree to which diploidy may be retained or, conversely, the rate at which aneuploidization may proceed during *in vitro* proliferation of normal derivatives varies from species to species (text-fig. 1). Included in this text-figure, based on earlier observations by Green and Todaro (*2*), are the trends noted for the Chinese and Armenian hamster embryonal and adult derivatives initiated in this laboratory (*3*, *4*).



TEXT-FIGURE 1.— Relative stability or retention of diploidy of fibroblast-like derivatives of different species [adapted from Green and Todaro (*2*)]. *Asterisks* indicate the status of inbreeding for each species.

One of several patterns of growth and karyotypic trends may be associated with the majority of routine isolates from different species. Examples are: *a*) Diplodi cultures of the Armenian hamster terminate abruptly within 4 passages in the absence of any selection toward aneuploidy (*5*); *b*) Syrian hamster and mouse cultures decline moderately or rapidly, while heteroploidization, with or without selection for a marker subline, characterizes surviving cell lines (*2*, *6–9*); *c*) human cultures remain diploid for some 50 passages before declining rapidly while displaying a wide assortment of karyological disturbances (*10–13*); and *d*) Chinese hamster cells (parent and clonal sublines) continue to proliferate rapidly while retaining diploidy for varying numbers of transfer generations (*ca.* 20–75), at which time near-diploid sublines emerge bearing numerical and/or structural alterations involving both autosomes and sex elements (*14–19*). Those in the audience more familiar with growth patterns of cultures from the rat, rabbit, cow, and pig are asked to assess our judgment in placing them in a position intermediate between human and Chinese hamster cells and also, perhaps, to comment later on the present status of inbreeding of each of these species.

With aneuploidy progressing rapidly during attempts to establish fibroblast-like derivatives of the mouse and Syrian hamster, there is much less possibility for a diploid tumor to arise following the inoculation of cells into appropriate hosts. The extent to which aneuploidy *per se* contributes to the establishment of permanent cell lines, with or without neoplastic tendencies, is discussed elsewhere in this monograph. Tests for neoplastic properties of human diploid cells in heterologous systems may, in some ways, be self-limiting, because of the need for constant repression of the hosts' immunological response for many months. However, until someone describes positive instances of neoplastic tendencies for primary cultures in a syngeneic rodent system, the negative findings for diploid human derivatives implanted into heterologous hosts will continue to remain unchallenged (*20*).

RELATIVE STABILITY OF DIPLOIDY IN NORMAL, NEOPLASTIC, AND VIRUS-TRANSFORMED CELLS

The role of a number of ill-defined species-specific factors in determining the relative stability or instability of the karyotypes of cultured cells, in general, is indicated in table 1. The assigned indexes were judged to be representative of observations made by others as well for each class of cells around the 40th transfer generation. Presently, the various species of hamsters are principal sources of tumor and virus-transformed cell lines which remain relatively stable. The low chromosome numbers of dwarf hamsters (Chinese and Armenian) encourage one to distinguish classic diploidy from minor types of pseudodiploidy. However, other parameters must be used in reaching a final decision. For example, growth rates and plating efficiencies of pseudodiploids, no matter how minor, are consistently 50–100% greater than corresponding clonal and parent diploid lines. Consequently, the indexes given in table 1 for dwarf hamster cells are, to the best of our judgment, indicative of classic diploids only.

Although the number of experimental species serving as sources of cultured cells is expected to increase with time, chromosome studies on cultured neoplastic and virus-transformed cells are, for the most part, presently limited to a few species—namely, man, mouse, Syrian hamster, rat, Chinese hamster, Armenian hamster, cow, and rabbit. The availability of syngeneic hosts to satisfy histocompatability requirements for tissue transplantation must be seriously considered when proposing a new rodent species for extensive use in cell-virus systems. Although the overall growth characteristics and karyological features of cultured tumor cells of the Chinese hamster have been conducive for attempts to associate minor chromosomal rearrangements and tumor progression (*4*, *21*), the results

TABLE 1.—Retention of diploidy in various cell systems (by the 40th transfer generation)*

Source	Normal	"Spontaneous" neoplasia	Virus-transformed (*in vivo* and *in vitro*)	Reference
Human	±	±	−	(*11–13, 25–49*)
Mouse	−	−	−	(*6, 7, 38*)
Rat	±	±	−	(*36–38*)
Syrian hamster	−	−	±	(*8, 9, 39–49*)
Chinese hamster	±	+	++	(*3, 4, 14–19, 21, 22, 24, 50*)
Armenian hamster	−	?	++	(*5*)
Bovine	±	−	−	(*51, 52*)
Porcine	±	?	?	(*53*)
Guinea pig	±	?	?	
Rabbit	±	?	?	(*54*)

*−, Aneuploidy predominates, if viable; ±, up to 50% are 2n (or pseudodiploid); +, up to 75% are 2n; and ++, 75% or more are 2n.

of these efforts were seriously dampened in the past because of the lack of syngeneic hosts. This one limitation has now been rectified by the recent development of two inbred lines now in the 28th generation of brother × sister matings. Trials with inbred lines are now under way, and the incidence of successful implantations among spontaneous tumors and viral-transformed cells has improved considerably. Consequently, the results reported herein for partly inbred, untreated and conditioned hosts (cortisone and/or 400 R whole-body X irradiation) must continue to be held in reservation, pending the outcome of future trials.

IN VIVO IMPLANTATION OF NORMAL DERIVATIVES

Since 1957, implantations of $3–6 \times 10^6$ cells into cheek pouches have included samplings from 30 embryonal and 30–40 adult lung derivatives. Implants have included primary cultures, cell lines (before and after the onset of aneuploidy), and several permanent cultures propagated in this and other laboratories for as long as 6 years. With the exception of the A_1 cell line, all others have been negative. Although repeatedly negative, *in vivo* testing has been continued in an effort to reconstruct the events leading to the formation of the exceptional A_1 cell line stemming from the third embryo culture initiated in this laboratory in 1957.

This particular embryo culture consisted of cells of both sexes, derived from a partly inbred female whose siblings and their subsequent litters developed a high incidence of spontaneous diabetes mellitus. By the 14th passage, sublines tended to segregate sexually, so that at implantation cell populations consisted predominantly of one sex or the other. A sarcoma (male) developed in only one cheek pouch which proliferated slowly upon transplantation. Pseudodiploid and near-diploid clonal sublines repeatedly isolated from tumor explants were equally effective in forming tumors (*22*). When cultured, cells displayed peculiar nucleolar buddings and extrusions (fig. 1), in the absence of elevated frequencies of chromosome loss or damage (*23*). More recently, we were informed by Dr. John Enders that this particular cell line exhibits an SV-like antigen.

This exceptional example of neoplastic transformation of a normal derivative took place at a time when oncogenic viruses were excluded from our premises. Since then, several oncogenic viruses have been used routinely, and yet control cultures and permanent cell lines of the Chinese hamster continue to lack neoplastic properties after many years of propagation.

In conclusion, the conspicuous nuclear budding patterns and detection of an SV-like antigen are offered as evidence to suspect the neoplastic tendency of A_1 male cells to have stemmed from some innocuous viral association which has yet to be repeated in our hands. The exact timing of this event cannot be determined: It may have occurred *in vitro* while cells were being cultured before the 14th passage, it may have occurred when cells were inoculated into the initial cheek pouch, or it may have occurred *in utero* involving one male embryo.

IN VIVO IMPLANTATION OF SPONTANEOUS AND RADIATION-INDUCED TUMORS

In sharp contrast to the infinite and rapid growth of normal and virus-transformed derivatives of the Chinese hamster, tumors (spontaneous, radiation-, cortisone-, and methylcholanthrene-induced) are exceedingly difficult to maintain both *in vivo* and *in vitro*. Less than 5% of the 150 or more tumors explanted during the past 10 years have proliferated *in vitro* as readily as normal and virus-transformed derivatives. Thirty-two of the more recent tumors were explanted simultaneously *in vivo* and *in vitro* (*3*, *4*). Of these, only 17 proliferated adequately enough in culture to enable chromosome checks and periodic implantations into cheek pouches (table 2). Ten (60%) were diploid (fig. 2), while the remaining 7 were either pseudodiploid or aneuploid (23–24 chromosomes). Three of the near-diploid karyotypes had conspicuous lesions or deletions at suspected secondary constrictions of chromosome 1 and the X chromosome. Only one-third of the ovarian tumors (4/12) cultured readily, whereas all 6 uterine carcinomas proliferated sufficiently during earlier passages to repeatedly note a uniform diploid state for both spontaneous and radiation-induced forms. The induction of pancreatic and adrenal tumors in animals stressed with cortisone may, perhaps, be a peculiarity of the Chinese hamster, which also develops spontaneous hereditary diabetes mellitus. Karyotypes of these rarer tumors were very similar to those observed for spontaneous and radiation-induced ovarian tumors.

TABLE 2.—Chromosome status of spontaneous and induced tumors of the Chinese hamster

Type of malignancy	Agent, if any	Number studied*	Chromosome status
Carcinomas:			
Ovarian	X ray (6)†	4/12	Euploid (2); pseudodiploid (1); and aneuploid (23 chromosomes with deletion of distal end of long arm of X's).
Uterine	X ray (3)†	6/6	Euploid.
Pancreas	Cortisone (1)‡	3/3	Aneuploid (24–27 chromosomes); pseudodiploid (modified 1 with deletion at point 7).
Adrenal	Cortisone (2)‡	2/2	Euploid; pseudodiploid (deletion of distal end of long arm of X's).
Sarcomas:			
Subcutaneous	3-MCA (4)§	2/9	Euploid.

**Numerator, number of tumors studied karyologically; denominator,* total number of tumors studied.

†Whole-body, fractional (3×200 R), or single exposure of 1000 R. Numbers in parentheses are the number of tumors that developed in the treated animals.

‡Subcutaneously, 25 mg.

§Wesson oil, 1 mg/0.1 ml.

Five diploid tumors underwent aneuploidization at varying times before the 52d passage. Comparisons of *in vivo* tests, before and after the onset of aneuploidy, suggested there was no apparent association between improved tumor progression and aneuploidy when limited to *minor* structural and/or numerical alterations, even though aneuploidization *per se* appeared to be reflected by slight improvements in growth rates (*4*). Drastic improvements in tumor progression have been observed only when aneuploidy has progressed, gradually or immediately, to the 3x modality. A similar pattern is now being witnessed among certain clones of polyoma-transformed diploid cells which, when placed *in vivo*, become unstable and approach the 3x modality by the third transplantation.

Only one exceptional tumor derivative has been isolated thus far from among the 150 or more tumors handled in this laboratory (fig. 3). Tumor 89, a "spontaneous" superficial sarcoma, biopsied at 4 months of age, gave rise to 5 primary sublines of which 4 have continued to proliferate rapidly. Growth rates contrasted sharply with the slower and more unpredictable manner in which other tumor derivatives have proliferated, even after the onset of aneuploidy. The initial biopsy of tumor 89 and all parent and clonal sublines implanted periodically over a 2-year period have failed to form tumors in animals. The latter situation is not uncommon for tumors of the Chinese hamster when syngeneic hosts are unavailable. What is considered uncommon is the continuous and extremely rapid proliferation that this exceptional tumor derivative exhibited from the very start. The following sequence of events is considered as being relevant to the latter point.

At the time of establishing the primary cultures, 5 separate flasks were seeded from a pool of trypsinized cells. Some 14 days later cells were observed to lift off the glass surface of one flask (C line), a condition which continued for 12 additional passages of this particular subline, during which time cyclic patterns of proliferation and degeneration took place. The remaining 4 cultures appeared unaffected. Upon passing the C-line supernatant and cellular debris to related sublines and normal embryonal derivatives, extensive cytopathic effects (CPE) developed gradually by the 12th day (figs. 4 a and b). A "carrier state" was repeatedly induced in one clonal subline, 11C, having an X0 karyotype. In such cultures, total cell destruction occurred only after infected cells were passaged 3 times (fig. 4c).

At no time during the 2 years of continuous propagation did the 4 remaining sublines and clonal derivatives exhibit any suspected signs of cell-virus activities unless infected with supernatants.

During the initial 12 months of study, characteristic "adeno-like" inclusion bodies were seen in all cultures innoculated with either supernatant, cell debris after freeze-thawing, or concentrations of virus after filtration and ultracentrifugation. Cell enlargement, extensive vacuolization, and formation of dense nuclear bodies (fig. 5a) paralleled inclusion patterns noted previously for certain strains of human adenovirus (*55, 56*). In "carrier-state" cultures of subline 11C, the degenerative process was much slower, as mentioned, and inclusion-body formation was preceded by telophasic arrest of cells having various levels of ploidy (figs. 5 b, c, and d). It appears that in "carrier-state" cultures, daughter cell formation was interrupted several times, thereby increasing the level of ploidy and susceptibility to CPE by the third passage after infection. The direction of cytoplasmic extensions (figs. 5 b and c) suggests the cells were still viable at the time of fixation and that the spindle mechanism was oriented along the short axis of the cell. Whether the latter is typical of normal mitotic events or the result of advanced CPE failed to be clarified.

The "adeno-like" CPE pattern and typical inclusion bodies ceased gradually by the 12th month, while giant cells with "foamy" cytoplasm increased in frequency (fig. 5d). It appears that the frequency of cultures developing CPE increased when the "foamy" or SV-like CPE pattern appeared (table 3). This latent change in the CPE pattern may indicate one of several interesting possibilities; *a*) viral mutation, *b*) activation of a second innate virus, or *c*) development of a "hybrid" virus.

Inoculation of newborn with supernatants before, during, and after the transition from "adeno-" to "SV"-like CPE failed to give rise to tumors during the 2 years of observation. Viral titers of fresh and frozen-thawed "adeno" supernatants were consistently low (table 4). In addition, storage at −40°F for 2.5 months lowered the titer considerably; *i.e.*, from 6×10^3 pock-forming units (PFU)/ml to 8×10^2 PFU/ml. Efforts to increase the concentration of virus by passaging onto cells of other species were equally ineffective. These tests, however, disclosed hamster cells, in general, were susceptible to both the "adeno" and "SV" supernatants. Embryonal derivatives of the Armenian and Syrian hamsters were affected equally with Chinese hamster cell types. In contrast, human cells (HeLa and WS amnion) and mouse cultures (embryo, conjunctival, and MFL) failed to display any signs of degeneration even after several passages, whereas green monkey kidney cells were positive when tested with "SV" supernatant.

TABLE 3.—Percentages of degenerating cultures and type of CPE after inoculation of 0.2 ml of fresh supernatant per 5 × 10^5 cells of clones 2A89, 4A89, 11C, and normal embryonal derivatives

Period (mo/yr)	Number of vessels treated	Number of vessels with CPE (%)	Type of CPE
12/62–10/63	329	252 (79.0)	"Adeno"
10/62–12/63	58	55 (95.0)	"Adeno-SV"
12/63– 3/64	120	113 (96.6)	"SV"

TABLE 4.—Plaque assay of "adeno-like" virus in fresh and frozen samples

Pool No.	Virus concentration	Number plaques/ 0.5 ml	Average	PFU/ml
64–1 (Fresh)	Undiluted	2220	—	4440
	10^{-1}	210 397	300	6000
	10^{-2}	31 54	43	8500
		(6×10^3 PFU/ml)		
64–4 (Frozen for 2.5 mo at −40° F)	10^{-1}	30	—	600
	10^{-2}	5	—	1000
		(8×10^2 PFU/ml)		

The exact role of the suspected agent, other than that of a passenger virus, must remain conjectural in the absence of similar "spontaneous" tumors. Nevertheless, the possibility that some "passenger" viruses may be implicated in the secondary transformation of tumor cells *in situ*, which, in turn, is reflected by exceptional growth characteristics *in vitro*, is of direct concern to us. The reason for our interest is that many of our cloned polyoma-transformed diploid-aneuploid sublines fail to evoke tumors when inoculated into cheek pouches, even though they exhibit morphological features typical of viral transformants.

IN VIVO IMPLANTATIONS OF POLYOMA- AND SV40-TRANSFORMED CELLS

Over 85% of the more than 150 clonal sublines isolated from mixed cultures exposed to polyoma virus and SV40 virus retain the diploid karyotype for periods ranging from 40–125 transfer generations (*3*, *4*). Unlike the control cultures that, in our initial experiments, remained diploid for only 30 passages, virus-treated cells exhibited morphological changes typical of transformed cells described for other species, but had spontaneous chromosome breakage frequencies that were 10–100 times less than controls. This stability in chromosome architecture of Chinese hamster cells before and, very surprisingly, after exposure to oncogenic viruses contrasts with similar studies employing primary-secondary cultures of other species (*8*, *26*, *31*, *37*, *49*, *67*). Such comparisons substantiate our thought that the relative stability of karyotypes of experimental cells is fundamentally determined by some ill-defined species-specific factor.

The tumor-forming capacity of 16 polyoma-transformed clonal sublines varied considerably after the inoculation of 3–6 × 10^6 cells into cheek pouches during the 3d–14th transfer generations (*4*). Three clonal sublines failed to produce tumors upon repeated inoculations (0/12 cheek pouches). Six others formed medium-sized tumors in only 9 of 33 cheek pouches, while the 7 remaining sublines formed large tumors in 13 of the 35 cheek pouches. *In vivo* trials were repeated when aneuploidy developed in each of the sublines. The comparative data suggested: *a*) The tumor-forming capacity of a given clonal subline of polyoma-transformed cells is not influenced by *mino*r shifts to aneuploidy, and *b*) not all morphologically transformed cell lines have tumor-forming properties. Similar trials are now under way using highly inbred animals as sources of embryonal cultures and as hosts for transplantation. Thus far, rapid tumor progression has been noted for two tumors initially started from diploid clonal sublines. By the third continuous transplantation in cheek pouches, chromosome numbers had shifted to a 3x modality. Diploid and near-diploid (minor aneuploid) implants proliferated much more slowly *in vivo* while retaining their original karyotype. Sublines of morphological transformants lacking tumor-forming properties are implanted in syngeneic hosts periodically, in an effort to determine if and when they may acquire neoplastic tendencies as karyotypes become progressively more varied in number and form. The outcome of these trials will help to evaluate earlier data on tumor-forming properties of polyoma-transformed cells in unrelated hosts, which suggested *in vivo* tumor progression remains unaffected by minor karyotypic alterations.

As mentioned earlier, normal embryonal and adult fibroblast-like cultures of the Armenian hamster fail to proliferate beyond 3 or 4 transfer generations, or some 30–40 days of maintaining primary cultures. Cultures exposed to SV40 and polyoma viruses also fail, even after displaying morphological changes indicative of successful transformation by an oncogenic virus. Such cultures must be retained for long periods to encourage survival via selective proliferation of rare aneuploids. These patterns for Armenian hamster cells contrast sharply with the ease in establishing and maintaining permanent cell lines, normal and virus-transformed, from the Chinese hamster.

PHENOTYPIC PARAMETERS ACCOMPANYING LOSS OF NEOPLASTIC PROPERTIES OF ADENOVIRUS #12-INDUCED TUMOR DERIVATIVES

An obscure but highly interesting series of observations was witnessed on three separate occasions while propagating adenovirus #12-induced epithelial-like tumor derivatives. The end result was the loss of neoplastic properties and alterations of other phenotypic parameters when tumor cells reverted to a non-tumor-forming fibroblast-like morphology. The evidence, as a whole, suggested that reversion to the fibroblast-like form may be related, directly or indirectly, either to the release or repression of the adenovirus genome from sites of incorporation in the host cell.

Three tumors of the Armenian hamster (1A12, 2A12, and 3A12) reverted spontaneously to fibroblast-like cell types before the 20th passage. One year later, two other Armenian hamster tumors (4A12 and 6A12) were exposed to SV40 and polyoma virus. They also reverted to the fibroblast-like morphology, in the absence of CPE or other unusual conditions that could have resulted in an abundant loss of cells. More recently, a Chinese hamster tumor (8A12) also reverted spontaneously to the fibroblast-like state. Spontaneous revertant cell types, implanted into cheek pouches at levels of $4\text{–}6 \times 10^6$ cells, failed repeatedly to form tumors. In contrast, similar implants of SV40- and polyoma-induced revertants resulted in tumor formation. However, indirect fluorescent studies for the T antigen were negative in spontaneous revertants, while positive fluorescence for SV40 T antigen was noted in the SV40-treated cells. Similar tests for polyoma-treated cultures have yet to be conducted.

Diploid fibroblast-like revertants of adenovirus #12-induced tumor cells of the Armenian hamster do, however, proliferate rapidly and exhibit many of the qualities of a transformed cell, except they lack the neoplastic properties and T antigen seen in epithelial or parent cells. Cloning efficiency is twice that of normal diploid fibroblasts, but equal to that of SV40- and polyoma-transformed cells (table 5). Clones of crisscrossing, fusiformed cells become multilayered within 7 days after petri dishes are seeded with dilute suspensions.

While cells were epithelial-like, diploidy involved 60–80% of the population, and stable karyotypes were noted for 20 or more transfer generations (fig. 7a). Cells with varying degrees of balanced ploidy (4n and

TABLE 5.—Comparative relationship of phenotypic parameters of adenovirus #12-induced epithelial-like tumor derivatives, spontaneous revertants, and normal fibroblast-like cells

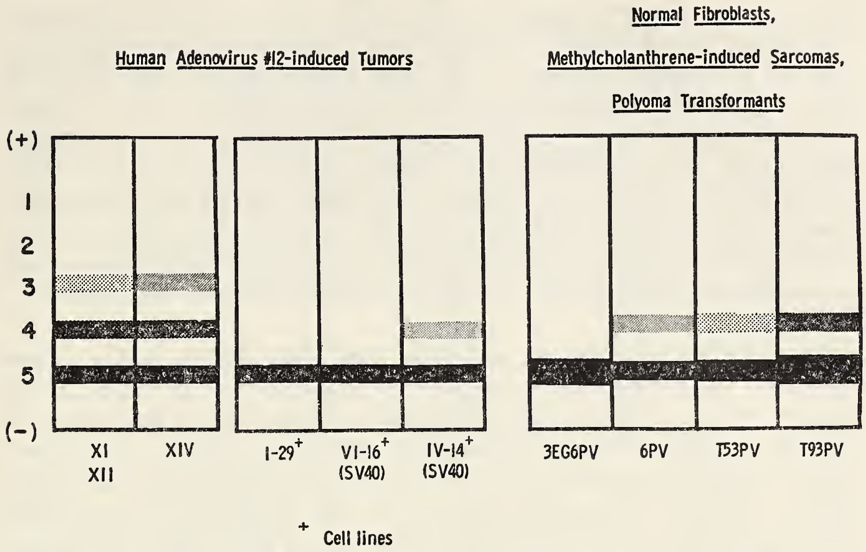
	Diploid cell types		
		Adenovirus #12-induced tumors	
Features	Normal fibroblast-like	Epithelial-like	Spontaneous revertants (fibroblast-like)
Retention of diploidy	+++	++++	++
Degree of aneuploidization	++	+	++
Cloning efficiency (%)*	10–20	0	20–40
Neoplastic tendency	−	+++	−
LDH isozyme patterns	4–5 and 5	3–4–5 only	4–5 and 5
T antigen	−	++++	−
Reciprocal chromatid exchange (X chromosomes)†	−	++	−
Prominence of secondary constriction (X chromosomes)	+	++++	+
Slenderness of late-replicating chromatin at metaphase	+++	−	+++

*Except for Armenian hamsters.
†Except for Chinese hamsters.

6n) were frequent in most sublines. Since endoreduplication was infrequent, such cells may have resulted from fusions. Cell aggregation or clumping noted after each trypsinization could have led to such events (fig. 7b).

While epithelial-like, tumor and cultured derivatives consistently displayed a -3-4-5 lactic dehydrogenase (LDH) isozyme banding pattern (*57*), as illustrated for tumors 11A12, 12A12, and 14A12 (left side of text-fig. 2). Spontaneous revertants to the fibroblast-like form, such as cell line 1–29 of tumor 1A12, have only the -5 isozyme band. The LDH isozyme patterns for the revertant sublines of tumors 4A12 and 6A12, isolated following exposure to SV40, were reduced to -5 and -4-5, respectively. With most normal fibroblasts and methylcholanthrene-induced sarcoma derivatives exhibiting only the -5 isozyme band and very few polyoma-transformed fibroblast-like derivatives featuring the -4-5 banding pattern (right side of text-fig. 2), the presence of the -3 isozyme in epithelial-like cells may indicate the adenovirus genome. Conversely, absence of the -3 isozyme reflects the release or masking of the viral genome in fibroblast-like revertants which also lack the T antigen.

The epithelial-like derivatives of tumors 1A12, 2A12, and 3A12 exhibited relatively high (but sporadic) instances of spontaneous reciprocal chromatid exchanges between X chromosomes specifically at prominent secondary constrictions on the short arms (*58*). Such events are rare in fibroblast-like derivatives and none have yet been observed among the revertants, which also feature poorly defined secondary constrictions or gaps on the



TEXT-FIGURE 2.—Comparative LDH isozyme banding patterns of adenovirus #12-induced (epithelial) tumors, spontaneous and SV40-induced (fibroblastic) revertants, and various fibroblast-like derivatives of normal, neoplastic, and viral-transformed origins.

short arms of sex elements. The more slender appearance of late-replicating segments of sex chromosomes typical of fibroblast-like cells was observed only after cells had reverted. Efforts to retransform revertants with adenovirus #12 have failed.

POSSIBLE ROLE OF NONONCOGENIC VIRUSES IN ESTABLISHING PERMANENT EPITHELIAL-LIKE CELL LINES

While we were conducting somatic cell fusions with ultraviolet-inactivated myxoviruses (*59*), primary embryo cultures of the Chinese hamster were exposed to live parainfluenza type-3 virus in an effort to understand more fully the response of dwarf hamster cells to this class of viruses. Within several days, fibroblast-like cells underwent CPE, leaving scattered islets of epithelial (renal?) cells to proliferate slowly as diploids for some 4–5 transfer generations. Thereafter, growth improved and the entire cell population was aneuploid (23–24 chromosomes). Implantation of 4–6 × 10^6 cells into cheek pouches of related animals failed to result in tumors.

Sublines were then exposed to human adenoviruses #7, 12, and 18 and SV40 virus. Within several transfer generations, cultures exposed to human viruses had temporary alterations in cell morphology and slight-to-moderate rates of CPE. Implantations into animals of the human adenovirus-treated cells were negative. The SV40-treated cultures responded immediately to the virus by undergoing telltale signs of giant-cell formation and a moderate amount of cell loss. Furthermore, when these cells were implanted into animals, tumors formed readily within 3 weeks. Histopathology of these initial tumors and subsequent *in vivo* passages were typical of nephroblastomas (Wilms' tumor), consisting of well-defined tubular elements composed of epithelial cells (*60*). The carrier state for myxovirus was confirmed for both tumor-forming and non-tumor-forming sublines by their patterns of guinea pig red-cell adsorption, which were similar to earlier results for human cell lines infected with myxovirus (*61*, *62*).

These preliminary studies suggested that some nononcogenic viruses may foster the development of permanent epithelial-like cell lines. In this instance where a carrier state prevailed, cell growth and karyotype remained virtually unaffected. Subsequent exposure to human adenoviruses failed to result in neoplastic transformation, while SV40 virus was highly effective. Failure to transform these renal cells with human adenoviruses may be indicative of several inherent restrictions, such as the lack of specific receptor sites for this class of oncogenic agents (in contrast to SV40). Another consideration which merits attention in future trials is the possibility that the carrier state for human parainfluenza type-3 virus, in some ways, interferes with the early phases of neoplastic transformation by human adenoviruses.

Properties of the exceptional cell lines of dwarf species of hamsters are summarized in table 6. Among the parameters recorded to date, the only direct association appears to be limited to the presence of a T antigen and tumorigenicity.

DISCUSSION

Are there underlying similarities hidden among the varied cell types of different species which are pertinent to the overall problems related to neoplastic transformation? Attempts to formulate common pathways for spontaneous and induced transformations have, invariably, included morphological, karyological, and antigenic parameters accrued from specific or known instances of cell-virus interactions. Similar approaches are now being extended to normal derivatives that have acquired neoplastic properties during *in vitro* progression, and to tumor derivatives, including sublines failing to retain neoplastic properties. Some investigators, particularly those in this laboratory, consider karyological and morphological approaches to distinguish among established cultures of normal, neoplastic, and virus-transformed cells, with and without neoplastic properties, as being totally misleading. Consequently, we have turned to antigenic approaches to assist in characterizing *exceptional* cell types more effectively.

Table 6.—Properties of exceptional cell lines of dwarf species of hamsters

Cell lines	Type of CPE	T antigen	Tumorigenicity	Type of growth	Chromosome status/sex
Embryo fibroblast, A_1	"Spontaneous," nuclear budding and extrusions.	SV-like	+++	Fibrosarcoma	22–27*/male.
Spontaneous tumor 89 (fibrosarcoma).	Adeno-like and later SV-like.†	?	—	—	22, 23/female.
Adeno-12-induced tumors:					
Epithelial-like	—	Adeno-12	++++	Small cell (lympho-) sarcoma.	22/both sexes.
Spontaneous fibroblast-like revertants.	—	—	—	—	22, 23.
SV40-induced fibroblast-like revertants.	—	SV40	++++	Fibrosarcoma	22, 23.
Parainfluenza type-3-treated:					
Renal derivative, P3	‡	—	—	—	23, 24/female.
P3 + SV40	SV40	SV40	++++	Nephroblastoma	23, 24.

*Clonal sublines.

†Agent isolated from 1 of 5 primary culture flasks.

‡Carrier state.

Normal, neoplastic, and virus-transformed cells of the Chinese hamster retain diploidy for comparatively long periods, in the absence of elevated frequencies of spontaneous chromosome breakage. When shifts in chromosome number and form do occur, most cloned and uncloned sublines tend to retain near-diploidy modalities. Difficulties in propagating neoplastic derivatives of spontaneous and radiation-induced tumors have restricted studies to karyological approaches only. Now, with syngeneic hosts available, prior transplantations of tumors *in vivo* may help in adapting some derivatives more readily to the *in vitro* environment.

A preliminary evaluation of a number of traits, such as the relative ease in propagating normal derivatives, degree of susceptibility of newborn to oncogenic viruses, capacity of cell systems to retain diploidy, and other pertinent features, places the Armenian hamster in an intermediate position, experimentally speaking, between the Syrian and Chinese hamsters. With inbreeding yet to be fully accomplished, all *in vivo* trials with the Armenian hamster must be reviewed with caution. Nevertheless, the low chromosome number, large sex elements, relatively high degree of diploid retention, and use of Armenian hamster cell types as parental forms for interspecific somatic hybrids (*59*, *63*) are but several of the traits currently being used in comparative studies with similar cell lines of the Chinese hamster.

Neoplastic transformation of normal embryonal derivatives of the Chinese hamster is, indeed, rare. The one exceptional A_1 cell line, isolated from a fibrosarcoma formed in a cheek pouch inoculated with diploid cells of the 14th passage, is strongly suspected to have acquired its neoplastic properties following a fortuitous encounter with an SV-like agent. Persistence of rarely observed nuclear and nucleolar buddings and extrusions suggests the agent is still present.

In contrast to this rare event among cell lines of the Chinese hamster, a rather consistent pattern is noted for the onset of neoplastic transformation during the course of propagating normal derivatives of the mouse and Syrian hamster (*see* Sanford, pp. 283–287). Thus, generally predictable traits recorded for derivatives of one species may prove to be exceptional among similar derivatives or cell types of other species. On the other hand, the limited proliferative capacity of normal diploid and SV40-transformed human fibroblast-like cells has yet to be reported in other cell systems.

A small percentage of the aged normal and X-irradiated (3 × 200 R) female Chinese hamsters develop tumors. These tumors are confined primarily to the uterus and ovaries. Males are extremely resistant to spontaneous and radiation-induced tumors. Thus far, the incidence of solid tumors among the 30,000 normal males maintained up to 8 months of age is 0.01%. A similar incidence has been noted for some 200 males maintained at varying times for as much as 5 years. Leukemia has yet to be observed. When coupling this data with the extremely low incidence of spontaneous neoplastic transformations of normal derivatives, identification of an inherent form of oncogenic virus, if any are present, will continue to be difficult.

Rapid, long-term proliferation of tumor 89 derivatives continues to be more impressive as new tumors fail to be established *in vitro*. Since the only other feature of this exceptional tumor derivative was the presence of a hamster-specific, "adeno-like" agent, the proliferative attributes of this cell type may have stemmed from a secondary *in situ* transformation by this nononcogenic passenger virus.

Events leading to the transition of adenovirus #12-induced epithelial-like tumor derivatives to the non-tumor-forming revertant fibroblast-like types are of considerable interest for a number of reasons. The question as to whether the adenovirus genome is actually released from its site of incorporation or, alternatively, inactivated by some repressor mechanism characteristic of only the fibroblast-like state of dedifferentiation, is fundamental to the overall problem of cell-virus interaction. When considering the former possibility, one must determine whether or not sites occupied by the adenovirus #12 genome are also points of integration for other types of oncogenic viruses. In planning experiments to test the second possibility, one must keep in mind that alterations from one cell type to another undoubtedly involve complex shifts in the repression-derepression mechanism, which, in turn, could lead to the masking of specific adenovirus incorporation sites, while revealing previously unavailable sites upon which other oncogenic viruses tend to interact more effectively.

Recently, Strohl *et al.* (*64*) described tumors arising from BHK 21 cells exposed to adenovirus #12 as epithelial-like, in contrast with the sarcomatous appearance of tumors formed by untreated cells. Our present studies support their conclusion that the adenovirus #12 genome determines the morphology of tumor cells.

Diploid fibroblast-like spontaneous revertants from adenovirus #12-induced tumor derivatives and diploid fibroblast-like sublines of tumor 89 are nononcogenic and exhibit many of the morphological features previously described for both nononcogenic and oncogenic polyoma- and SV40-transformed cell lines. With most normal or control cultures, other than the exceptional A_1 derivative described above, failing to express or acquire oncogenic properties after propagating for as long as 6 years, and with spontaneous fibroblast-like revertants of adenovirus #12-induced tumor derivatives failing abruptly to express oncogenicity with loss of the T antigen, a rather rigid line of demarcation appears to separate nononcogenic from the oncogenic cell lines of the dwarf species of hamsters. The immediate difference has now been reduced to the presence or absence of an appropriate T antigen. In addition, the status of the viral genome also may be indicated morphologically, enzymatically, and chromosomally, as observed for the adenovirus #12-induced tumor derivatives.

Untreated or control fibroblast-like cultures of the mouse and Syrian hamster tend to acquire neoplastic properties at varying times after being established (*9*, *65*). A related series of events was reported by Diamandopolus and Enders (*66*) for cell lines isolated from SV40-treated lung and liver cultures of newborn Syrian hamsters. Although the latter cell lines displayed no apparent association with the viral genome, many eventually acquired neoplastic properties after the 23d transfer generation. In essence, these recent examples of "spontaneous" malignant transformations are reminiscent of the trends noted previously for the BHK 21 cells and similar derivatives of the Syrian hamster (*39*, *40*, *43*).

In contrast to the trends reported by others for normal fibroblast-like derivatives of the mouse and Syrian hamster, the incidence of "spontaneous" neoplastic transformations of normal derivatives of the Chinese hamster is estimated to be less than 1%, when considering sublines established from the 60-odd explants initiated in this laboratory during the last 10 years. The gain and loss of proliferative and neoplastic properties of exceptional normal, neoplastic, and virus-transformed cell lines of dwarf species of hamsters have been, directly and indirectly, associated with the activities of known and unknown viruses. Favorable properties of the Chinese hamster are: ease in establishing rapidly proliferating, normal fibroblast-like derivatives, long-term retention of diploidy, availability of inbred lines, simplicity of the karyotype, ease in obtaining viable forms of hybrid cells from somatic fusions, and overall sensitivity of cultured cells to oncogenic viruses. The predictable stability of cell types and relatively low frequency of spontaneous neoplasia are expected to be highly useful for designing more precise studies regarding cell interactions and oncogenicity after exposure to viral, chemical, and physical agents. With diploidy predominating among clonal cell types, alterations in the various proliferative and metabolic parameters resulting from cell-agent interactions may readily be ascertained before the onset of similar, but secondary, changes that accompany aneuploidization *per se*. The suitability of cultured cells of dwarf species of hamsters for viral vaccine production has yet to be determined.

SUMMARY

Several exceptional diploid cell lines of the Armenian (*Cricetulus migratorius*, $2n = 22$) and Chinese (*Cricetulus griseus*, $2n = 22$) hamsters exhibited growth and cellular features that differed strikingly from similar cell types routinely isolated in this laboratory during the past 10 years. Among the 60-odd normal embryonal and adult derivatives, only the A_1 cell line has acquired neoplastic properties. Nuclear budding and presence of an SV-like antigen suggested that the "spontaneous" transformation stemmed from a fortuitous exposure to a viral agent. Thirty-two of the more than 150 spontaneous and radiation-induced tumors of the Chinese hamster were placed simultaneously *in vivo* and *in vitro*. Only one spontaneous diploid tumor, tumor 89, has proliferated extensively *in vitro*. A

nononcogenic, hamster-specific "adeno-like" virus was isolated from one primary subline. Proliferative features of this tumor derivative may have stemmed from a secondary *in situ* transformation by the "adeno-like" passenger virus. Several adenovirus #12-induced epithelial-like tumor cell lines have reverted to fibroblast-like forms, spontaneously and following SV40-exposure. Spontaneous revertants were tumor-negative and lacked the T antigen, whereas SV40-induced revertants had only the SV40 T antigen and formed fibrosarcomas. A permanent epithelial-like (renal?) cell line was isolated from embryonal cultures exposed to parainfluenza type-3 virus. Cells were non-neoplastic and reflected a virus carrier state. After transformation by SV40 virus they gave rise to tumors resembling neophroblastomas. In the cases studied, exceptional properties of each of these cell types appeared to be directly related to the presence or absence of the appropriate T antigen.

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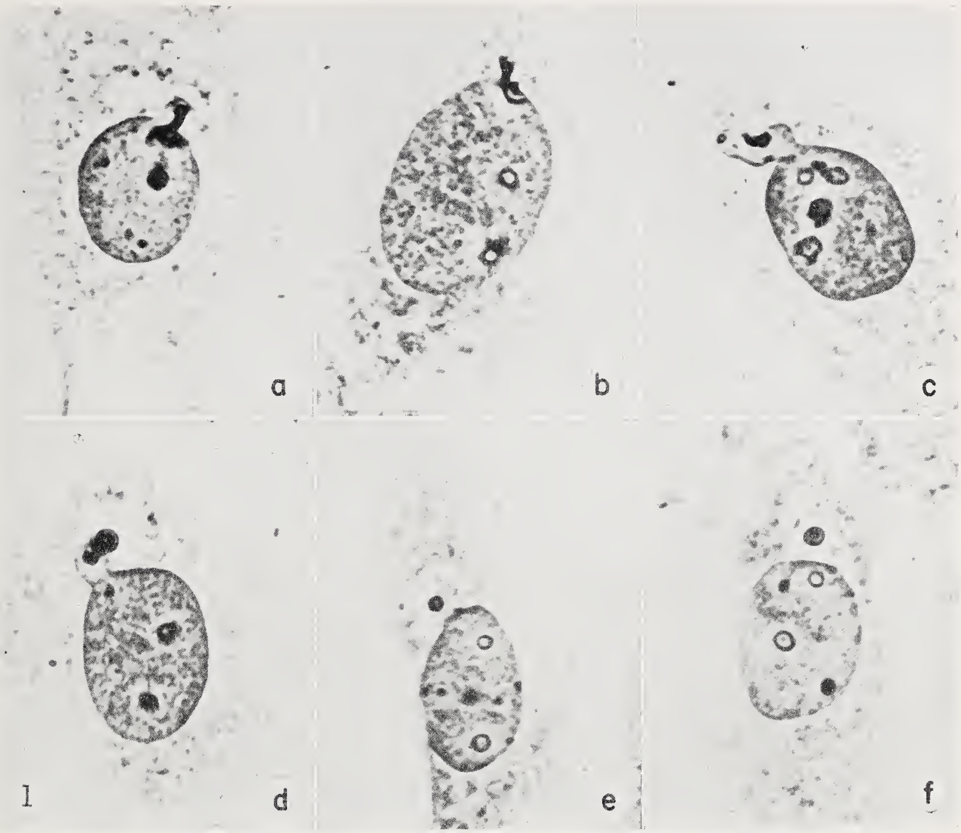
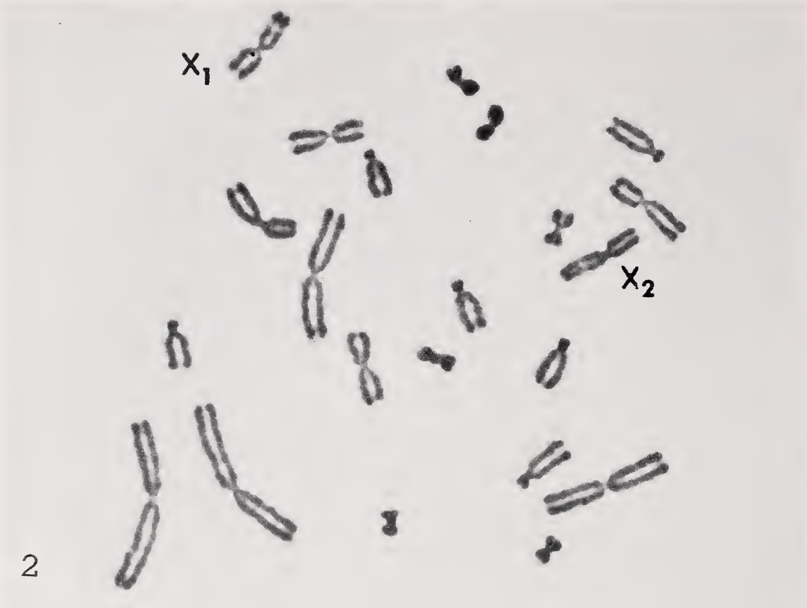


FIGURE 1.—Reconstruction of nuclear budding and extrusion phenomena noted specifically in A_1 cells derived from "spontaneous" neoplastic transformation of a male embryonal derivative. (a, b) Selective extrusion of nucleolus or chromatin mass from the nucleus; (c) an example of finger-like nuclear extension, followed by (d) selective extrusion of nucleolus or chromatin mass, with retraction of the nuclear sap; (e, f) severance of bud, closure of the nuclear pore, and rounding-up of the extruded material in a cytoplasmic clear zone. Formalin fixation. Hematoxylin and eosin.



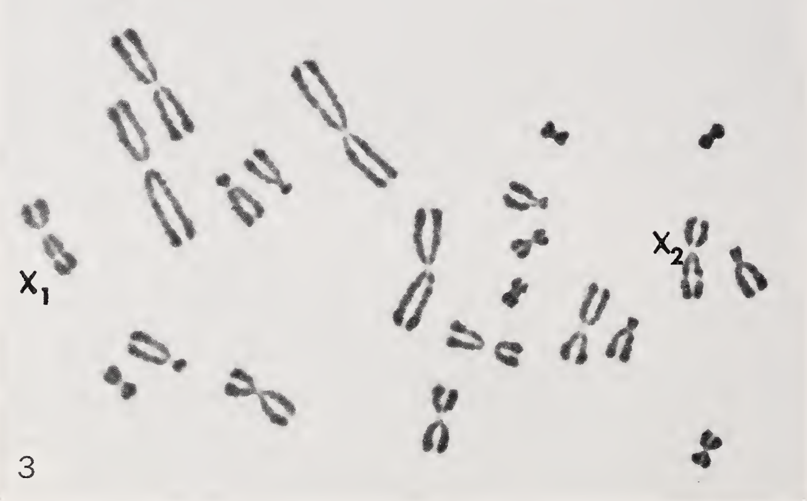


FIGURE 2.—Karyotype of uterine tumor T100. *Note* characteristic secondary constrictions on the long, late-replicating arms of the X chromosomes. Transplantable.

FIGURE 3.—Karyotype of "spontaneous" Sarcoma (tumor 89) (clone C4A)· Diploidy persisted for 50 transfer generations. Chromosomal features are very similar to those of other diploid tumors, viral-transformed cells, and normal derivatives. Not transplantable.

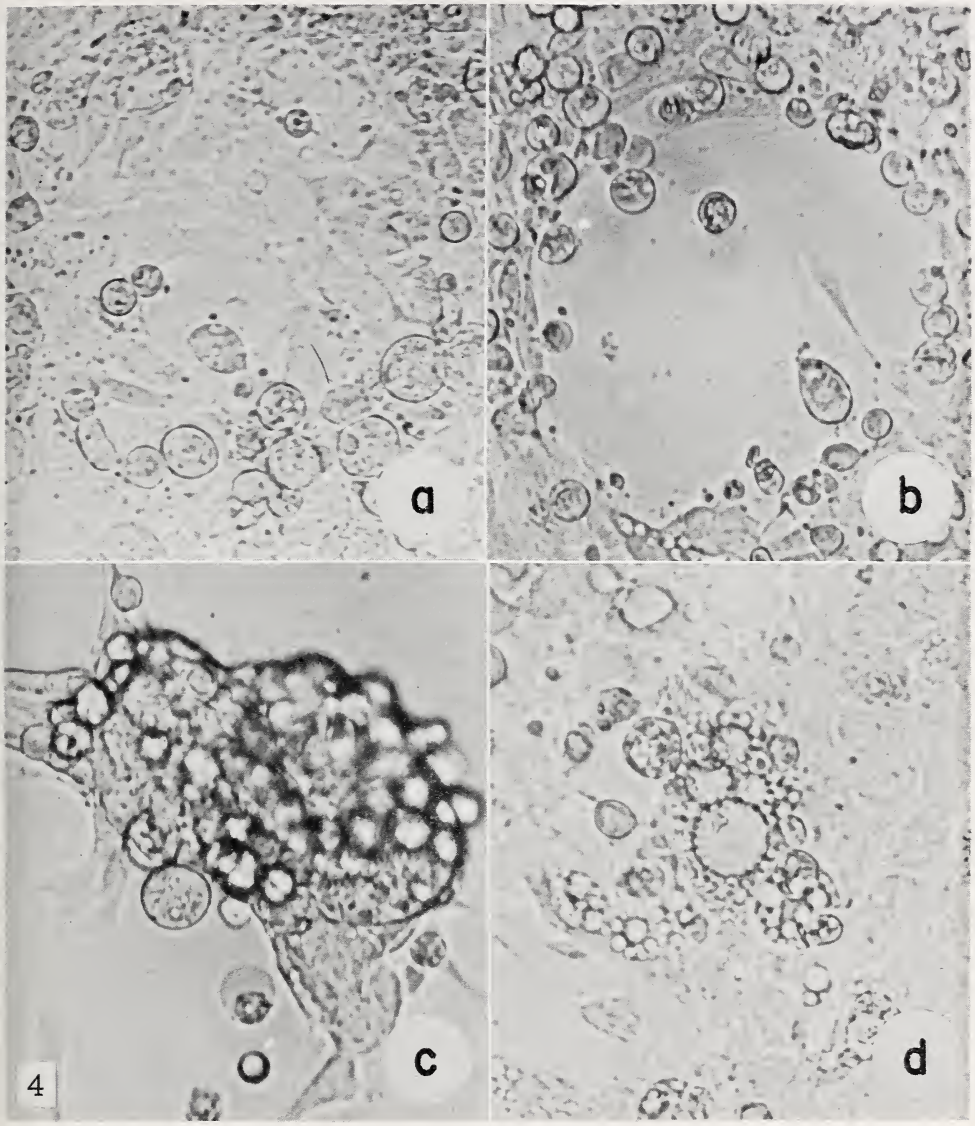


FIGURE 4.—Cytopathic effects on clonal sublines inoculated with 0.2 ml supernatant initially taken from the C line of tumor 89. (a) Formation of a degenerating focus around 5th or 6th day after infecting normal embryonal and clone 4A cells; (b) enlargement of focus by the 10th or 11th day after infection; (c) massive clumping of clone 11C cells 3 passages after inoculation: (d) appearance of cells displaying "foamy" cytoplasm 12 months after continuous propagation and reinfection of clone 4A cells with supernatant. The characteristic production of foci (b) and "adeno-like" inclusion bodies (figs. 5a–d) ceased when "foamy" cytoplasm dominated infected cultures.

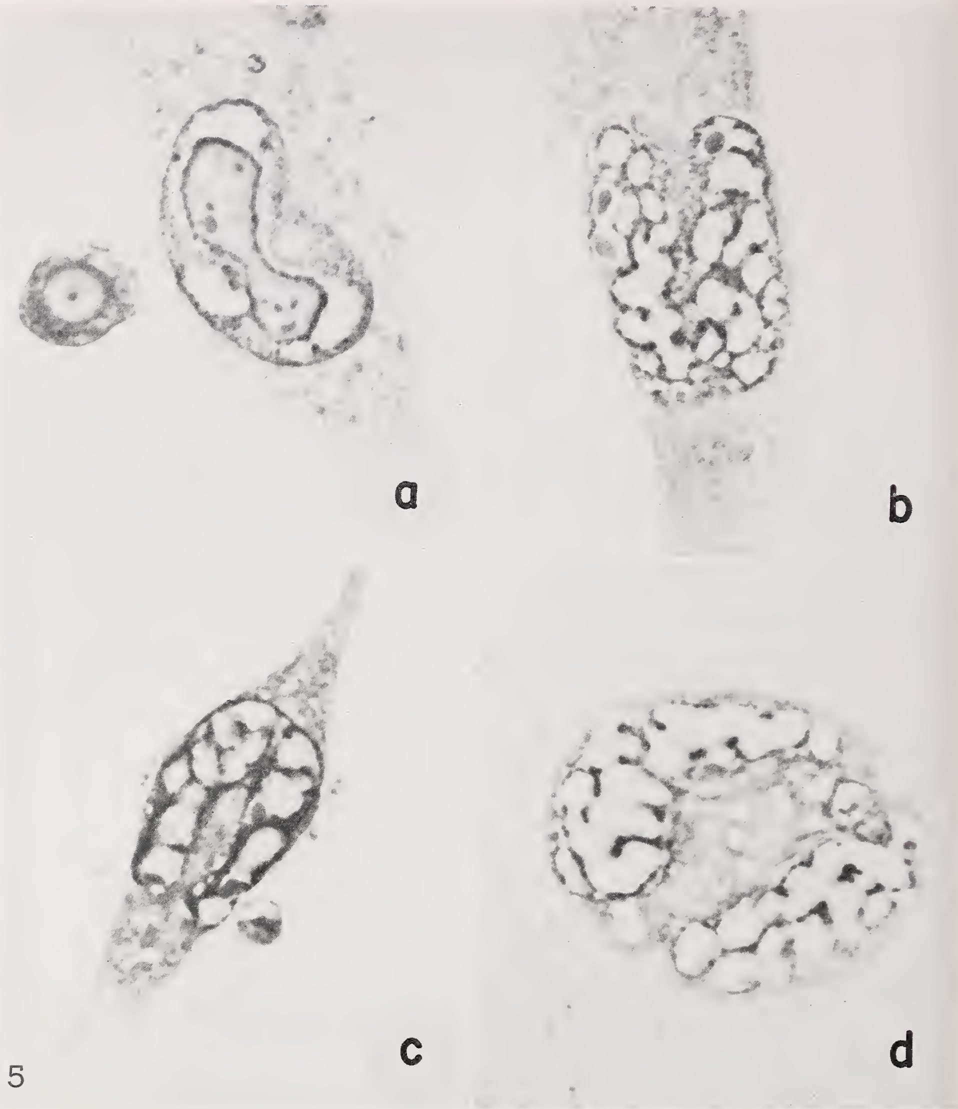


FIGURE 5.—Examples of "adeno-like" inclusion bodies in cells following inoculation of supernatant from C line cultures of "spontaneous" tumor 89. (a) Clone 4A cells of varying size (ploidy?) featuring retraction of chromatin, vacuolization within nucleus containing scattered, dense, chromatic bodies; and (b–d) examples of telophase-arrested polyploid cells in carrier-state cultures of clone 11C showing early signs of CPE: failure of daughter cell formation, margination of chromatin, and formation of dense chromatic bodies. Appearance of cytoplasmic projections and nuclei (b, c) suggests spindle mechanism may have been oriented along the short axis of the cell.

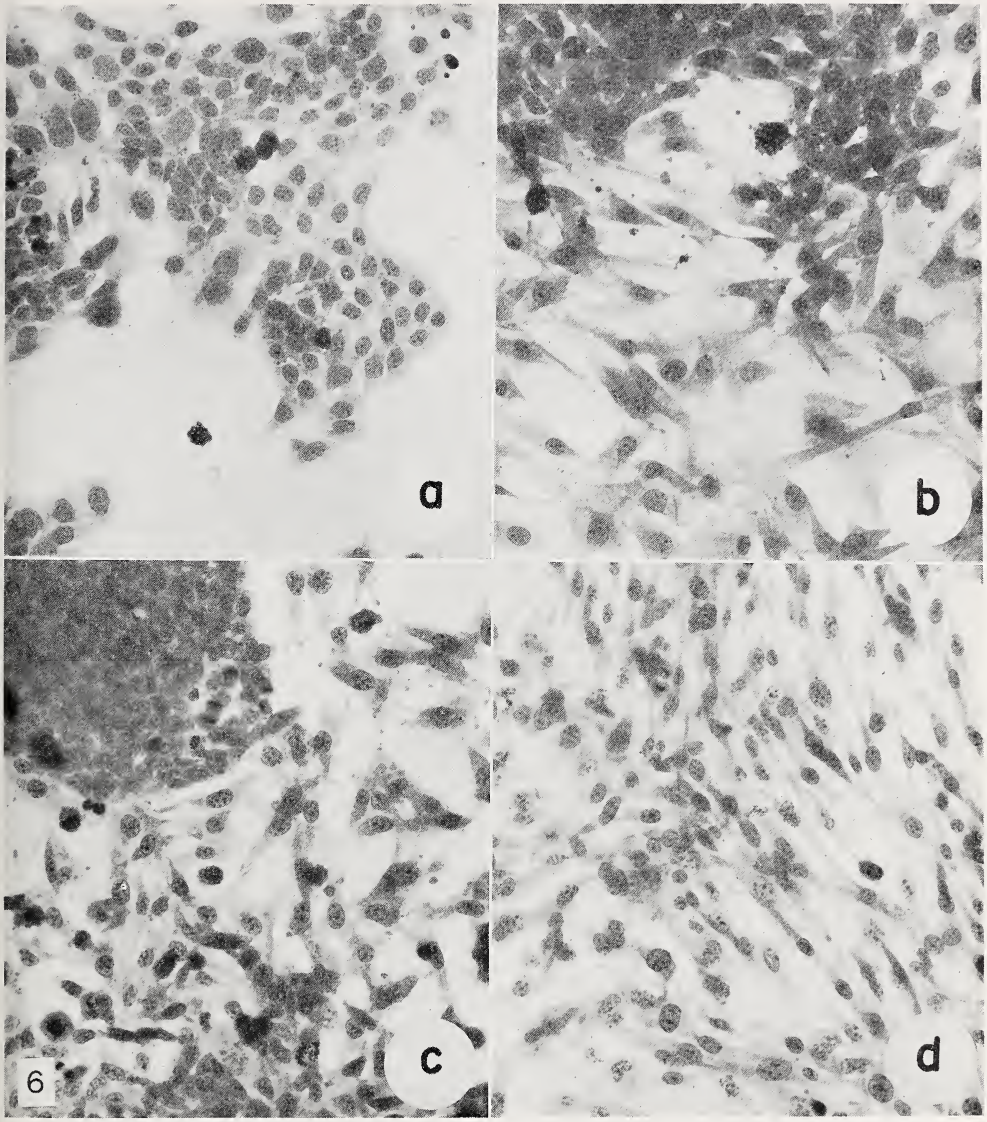


Figure 6.—Progression of spontaneous and induced revertants of epithelial-like adenovirus #12-induced tumor derivatives (tumor-forming) to fibroblast-like cells (non-tumor-forming). (a) Characteristic appearance of cultured epithelial derivatives of tumors induced in both the Armenian and Chinese hamsters; (b) rapid development of fibroblast-like forms (within 48 hours after exposure to SV40) stemming from epithelial-like parental clusters; (*c*) spontaneous reversion to fibroblast-like forms during 5–8 transfer generations of 9 sublines of tumors 1A12, 2A12, and 3A12; (d) final and persisting appearance of spontaneous and SV40-induced reverted sublines.

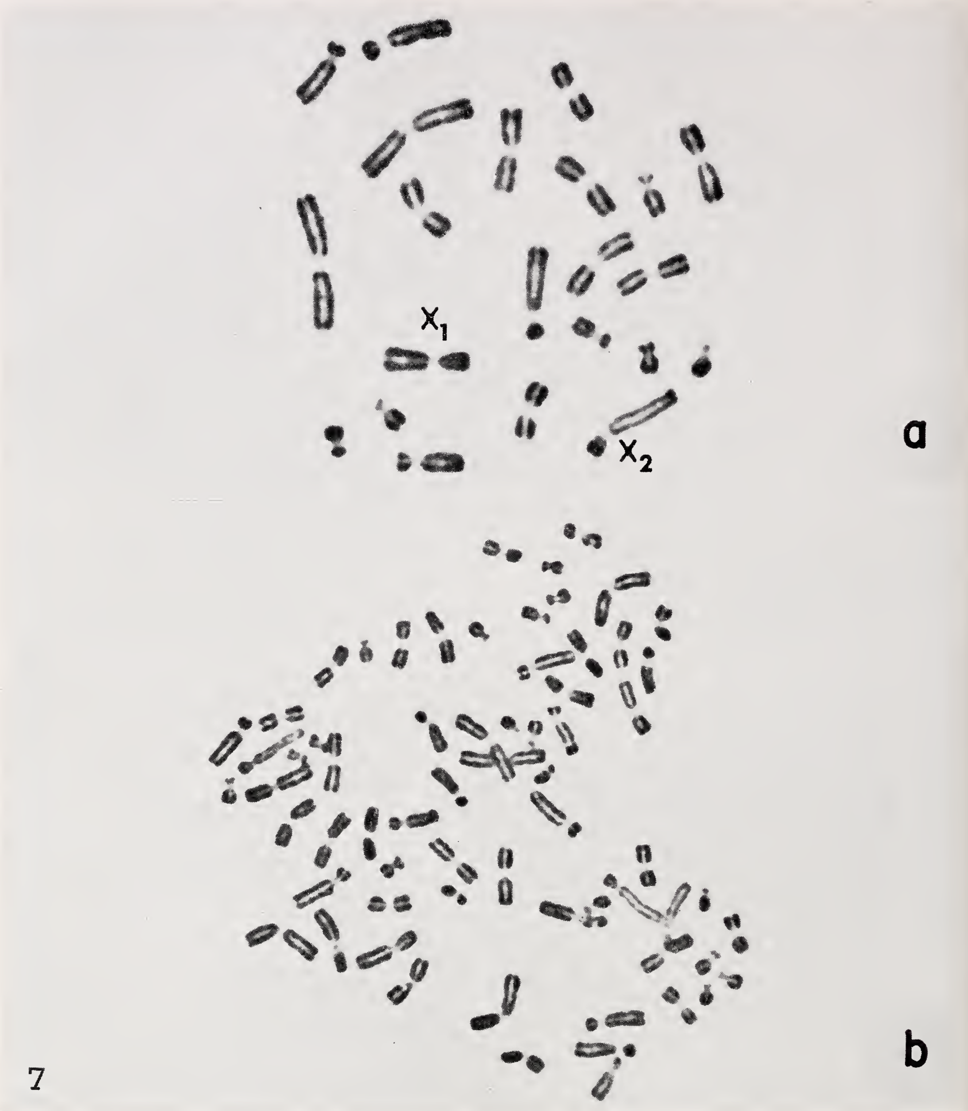


FIGURE 7.—Euploid karyotypes of human adenovirus #12-induced epithelial-like tumor derivatives. (a) Stable diploid (2n = 22) chromosome complement of tumor 1A12. *Note* distinctive heteromorphism and prominent secondary constrictions on the short arms of the X chromosomes; (b) balanced hexaploid arising presumably from fusion of 2n and 4n cells.

Evidence for Oncogenic Properties of Primary Cultures

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It is a well-known fact that cell cultures from embryonal tissues of several species upon continuous growth *in vitro* eventually change in several characteristics—morphological, karyological, and immunological—and can produce tumors when inoculated into suitable hosts.

There are two possible explanations for this phenomenon: 1) A small fraction of abnormal (tumorigenic) cells may be present in the initial population, although their presence may not be detected until progressive subcultivation and possible alteration of intercellular growth controls allow their multiplication; or 2) the "spontaneous transformation" may be a late event which can be imposed from without or may originate, possibly by mutation, within the normal cell population.

Whichever of these two alternatives is correct, it becomes very cogent to determine whether even a normal cell population constitutes a risk when inoculated into a potentially susceptible host.

We have investigated this problem by taking advantage of an old observation that embryos, transplanted into adult animals, hamsters, mice, or rats, continue to grow to form "embryomata."[1] Hamster embryos of different age from 8–16 days of gestation were fragmented or smashed and inoculated at the dose of 2–4 embryo equivalents in the subcutaneous or intramuscular tissue of adult hamsters. Within 10 days palpable nodules invariably appeared. They grew progressively to reach 1–1.5 cm and persisted as long as the animals were kept under observation, as long as 9 or 10 months (table 1).

On histological examination, the nodules consisted of tissues representing all the 3 germinal layers with no evidence of abnormal cells. The growth in size was due not only to the formation of cysts but also to actual proliferation of cells either in organotypic structures or in a randomized fashion.

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[1] This work was conducted in collaboration with J. Lehman.

Table 1.—"Embryomata" formation by hamster embryonal tissues

Inoculum	Age of embryos (days)	"Tumor" incidence	Persistence (mo)	Maximum size (cm)
Embryo mash	8	12/13	3	1
	11	59/60	9	2
	13	10/10	9	1.5
	14	7/7	9	1.5
	15	4/6	9	0.4
	16	4/4	3	0.6
Intact embryos	11	3/4	6	1.3
	12	5/5	9	2.5
*Trypsinized embryo cells:				
3×10^7	11	3/4	2	0.3
8×10^6	11 + 4†	0/4	—	—
1.2×10^7	15	0/5	—	—
0.9×10^7	15 + 1*	0/4	—	—
1.5×10^7	15 + 1	0/4	—	—
0.6×10^7	15 + 2*	0/4	—	—
1.5×10^7	15 + 2	0/4	—	—

*Inoculated into 5-day-old animals.

† Days after "+" sign indicate period of time *in vitro* after trypsinization.

We verified that there was actually continuous growth of cells under these conditions by doing incorporation studies with ^{3}H-thymidine. However, neither invasion of surrounding tissues nor metastasis was observed. When we transplanted several of these nodules at various times after initial implantation—and we tested about 20 of these—no further growth occurred in the secondary transplant. Also no growth occurred when a number of cells equivalent to those in an embryo—*i.e.*, about 30 or 40 million cells—were inoculated after trypsinization.

These experiments tend to exclude the possibility that a small fraction of tumor cells may have already been present in the embryonal tissues, since they could not be detected under this type of selective *in vivo* conditions.

Therefore, primary cells *in vitro* and, by inference, cell cultures which have the properties of primary cells should not be considered to have an intrinsic tumorigenic risk, at least by the criterion of transplantation. The greater tumorigenic risk of primary culture is that of containing latent tumor viruses, possibly in a defective state. This risk cannot be underestimated, since viruses oncogenic for some species may be cytopathic for others and cannot be identified from any characteristic features. In this sense, a few "normal" lines well characterized would constitute less of a danger for vaccine production than the use of primary cultures.

We have to accept the fact that whenever active biological materials are administered in a large population some risks are involved. With the evidence we have up to now, the risk that "normal" diploid cultures may be tumorigenic is the least of the worries we have.

Variable Susceptibility of Human Cell Strains to SV40 Transformation

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Human diploid cells growing in culture can be transformed by SV40. We have developed and studied an assay system that measures the fraction of cells in a given population that become transformed and have compared the susceptibility of normal cells derived from various human sources to SV40 transformation (*1*, *2*).

The most susceptible cells found so far have been derived from patients with Fanconi's anemia, a rare autosomal recessive disease that is associated with a high risk of tumor formation (*3*). Cell strains from these individuals also show a high level of chromosome breakage that persists throughout the *in vitro* lifetime of the cell culture.

However, despite this high level of chromosome breakage, the uninfected cultures do not evolve into permanent cell lines. Instead, they die out after roughly 50 cell generations just as normal cells do.

This finite lifetime in culture is also true of strains derived from patients with trisomy 21 and trisomy 18 syndromes. Although the chromosome abnormality persists as long as the cultures are carried, the cells die out at the expected time. The trisomic cells like the Fanconi's anemia cells, however, also have an increased susceptibility to SV40 transformation.

Table 1 shows some of the data comparing adult skin fibroblasts from normals and from patients with Fanconi's anemia. The seven normals fall into a rather narrow range, showing roughly 2–5 transformed colonies per 10^4 cells. The Fanconi's anemia homozygous cells are 10–50 times higher. The Fanconi's anemia heterozygous cells also are considerably higher. If we use E46, an adeno-7 SV40 "hybrid" virus, it also appears that the Fanconi's anemia cells are of the order of 20–40 times higher.

Table 2 summarizes the data presently available. The 12 embryonic lung strains tested have a low or normal transformation susceptibility, showing less than four transformed colonies per 10^4 cells at a multiplicity of 1,000. WI–38 was among the strains tested.

Table 1.—Transformation frequency of virus-infected human cell lines (values are means, ± standard errors, of 2–6 separate experiments, in each of which a total of at least 2 × 10^5 infected cells were plated)*

Cell strain	$\frac{\text{Transformed colonies}}{\text{Cells plated}} \times 10^4$	
	SV40†	E46‡
Normal adults		
A	4.4 ± 1.1	0.8 ± 0.3
PB	1.6 ± 0.2	
JW	4.3 ± 0.6	0.5 ± 0.2
LG	5.1 ± 0.6	0.4 ± 0.3
HG	2.6 ± 0.7	0.4 ± 0.2
LK	3.0 ± 0.5	
RT	3.7 ± 0.6	
Fanconi's anemia, homozygous		
AM	79.7 ± 18.1	16.3 ± 3.4
JV	41.4 ± 12.5	
Fanconi's anemia, heterozygous		
TM	20.1 ± 3.2	
CV	28.2 ± 8.7	

*Reprinted from *Science* 153, No. 3741, Sept. 9, 1966.
†Infected with $10^{8.0}$ TCID/ml.
‡Infected with $10^{8.2}$ TCID/ml.

Table 2.—Transformation susceptibility of human cell lines

Source	Number tested	Number with increased susceptibility
Embryo lung	12	0
Newborn foreskin	8	0
Adult skin	17	1
Aged adult skin	4	1
Down's syndrome	5	5
Fanconi's anemia:		
Homozygous	4	4
Heterozygous	5	5
"Sarcoma-prone" family	5	3
"Transformation-prone" family	3	3

The eight cultures of newborn foreskin we have tested also are normal. Of the 17 adult skin cultures, one from an apparently normal individual was quite high; but a look at the last line reveals that three relatives of this individual, two siblings and his father, also are very high. So this appears to be a genetic property of the cells obtained from this particular family. Of fibroblast strains derived from five members of a family in which several cases of sarcoma have occurred, three strains show exceptionally high transformation susceptibility.

All the Fanconi's anemia cells we have tested, homozygous and heterozygous (only 9 cases), are high. All of the Down's syndrome (trisomy 21) cells are also out of the normal range. In the cases studied so far the susceptible strains also show a much higher fraction of SV40 T antigen-positive cells 48–72 hours after infection when compared to the normal and the resistant strains (*4*).

If we compare late-passage material from any of these strains with early passage material, we find the late-passage material is more susceptible to transformation. Cells from elderly individuals, patients over 70, also show a higher susceptibility than their embryonic and newborn counterparts when tested as secondary or tertiary cultures. So there does appear to be an age factor that acts both in tissue culture and in the body. This change in susceptibility in culture occurs between the 20th and 30th cell doubling, and it occurs, at least in our hands, before we can detect any increase in chromosome abnormalities.

Cloned lines from these cells also are almost invariably more susceptible than the original mass population. This probably is because such a significant fraction of the human cell's lifetime is needed to grow up from a single cell to mass culture. Growth from a single cell to a million cells requires 20 cell doublings. So then, by the time it is tested, the cloned line already has an elevated transformation rate as compared to the rate found in primary and secondary cultures.

To summarize, then, human cells, even karyotypically abnormal human cells, do not spontaneously evolve into permanent cell lines. Karyotypically abnormal cells are more likely to be transformed than their normal diploid counterparts. However, the converse need not be true. There are chromosomally normal strains that do have a high susceptibility to SV40 transformation. The data, although still based on relatively few cases, suggest that the assay may be used to detect persons with an unusually high risk of developing tumors.

Finally, if one wished to look for oncogenic agents for human cells, the highly susceptible cells such as those derived from patients with Fanconi's anemia cells appear to offer the most sensitive *in vitro* test system.

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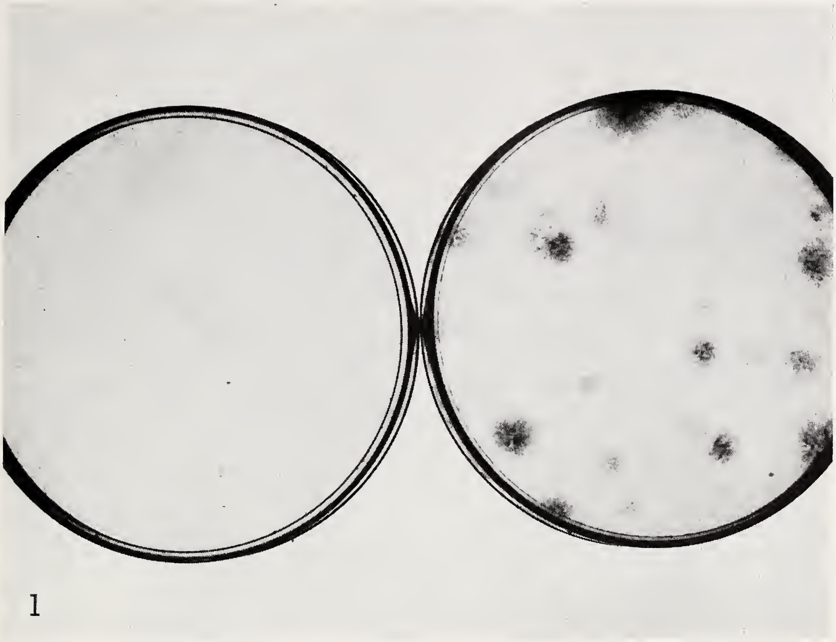

Figure 1.—*On left* a petri dish seeded with uninfected normal adult human fibroblast cells and *on right* cells that have been infected at a multiplicity of 1,000 and allowed to grow for 15 days before being fixed and stained. The transformed colonies were scored under low power with a dissecting microscope.

Multiple-Layered Perfusion Cultures

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IN recent years it has been implied frequently that one thing which distinguishes normal cells from neoplastic cells *in vitro* is that the former cease to proliferate upon confluency, whereas the latter pile up three dimensionally. There may be a quantitative difference between paired systems of the two classes of cells in this respect, but in general it certainly is not to the degree which some have assumed it to be, and Dr. Foley and Dr. Yerganian have already referred to this. As a specific example, SV40-transformed WI–38 cells pile up in culture faster, in our experience, than the parent WI–38. However, by simply perfusing cultures of diploid cells of finite lifetime *in vitro*, such as the WI–38, one can achieve multiple-layered populations having a "tissue-like" three-dimensional density. If one seeds culture flasks with diploid cells sufficient to cover 10–20% of the glass surface and then perfuses them, the cell population proliferates to confluency in several days, whereupon the proliferation rate decreases but continues so that the equivalent of multiple layers of cells form in as little as 3–5 additional days of perfusion culture. We have observed this many times, for example, with WI–38 cells and also with the RPMI–2650, the Flow 2000 line, bovine cells from Dr. Alexis Kniazeff, the DON Chinese hamster cells, and a JAM cell initiated in our laboratories by Dr. Ed Miedema from the tonsil tissue of an 8-year-old boy; these last cells phase out at about 20 passages *in vitro*. As a side remark here, I would like to mention that in a recent experiment the JAM cells were allowed to proliferate to the equivalent of 2 layers and they were then maintained at a constant population for 12 days, at which time the experiment was stopped arbitrarily, with the influent perfusion medium containing 1% serum and 100 mg% glucose. Thus, extended maintenance of a dense population of diploid cells can be accomplished under perfusion conditions.

With regard to primary cultures, the only experiment we have done which might have a bearing was with a rat embryonic cell line put on perfusion at the third passage. This reached the equivalent of 10 layers of cells in $5\frac{1}{2}$ weeks of perfusion; the total number of passage equivalents was still less than 10, assuming proliferation from 1–2 layers, 2–4 layers, and 4–8 layers as each equivalent to one so-called passage (doubling). This culture was pulsed 90 minutes with tritiated thymidine, and subsequently the distribution of labeled nuclei was found quite uniform throughout; *i.e.*, from bottom to top. In two recent experiments with multiple-layered Jensen sarcoma and WI–38VA13A cells, patterns of labeled nuclei were quite different, being almost entirely in the upper layers and at the very top layer of the culture. This pattern perhaps is similar to the situation *in vivo* with transplantable tumors, in which proliferation is usually considered to take place mostly toward the periphery of the neoplastic mass.

I am quite biased regarding perfusion-type cultures; one gains so much better control over the culture environment that I wonder why some type of perfusing system is not used *in vitro* more than it is. Nutrient concentrations and *p*H can be held within narrow ranges and metabolic products are removed. Dense, multiple-layered populations of cells are easily obtained, and these tissue-like cultures can be examined for properties which may or may not characterize them as possessing tumorigenic or nontumorigenic properties.

Whether the output of virus from a multiple-layered diploid tissue culture might be increased over a monoconfluent sheet culture remains to be seen.

GENERAL DISCUSSION

Dr. P. Gerber: Dr. Todaro, would you clarify your comment on the lack of immortality of SV40-transformed Fanconi's cells versus the supposedly immortal SV40 form of the 3T3 type?

Dr. G. J. Todaro: I meant that the normal cells, the uninfected cultures, die out at the expected time. The transformed cells, of course, do become immortal. They go through the "crisis" phase, but then they come out of it and become permanent cell lines.

One might have thought that, if we started with a cell that was karyotypically abnormal from the very beginning, such a cell might have a higher probability of spontaneously transforming than a straight diploid cell. So we tried this with the Fanconi's anemia cells where there is a high level of chromosome breakage. We have also tried this with the trisomy cell where there is an extra chromosome. These cells seem to reach the phase-3 phenomenon at roughly the same time and die in the same way. So a high level of chromosome breakage is not sufficient to make human cells spontaneously evolve into permanent lines.

Dr. Gerber: Dr. Kruse, would you comment on the possible mechanism of perfusion to overcome contact inhibition in normal cells?

Dr. P. F. Kruse, Jr.: As I understand it, contact inhibition in the original context of Abercrombie and Ambrose referred to cell movement. It has been assumed in recent years that it also may signify cessation of proliferation upon cell contact. However, this connotation is not necessarily correct.

Now, under perfusion conditions, whether or not we are washing out some sort of cell inhibitor, I don't know. But at any rate, under these perfusion conditions, contact inhibition does not mean cessation of proliferation of diploid cells.

Dr. M. M. Sigel: I would like to make two comments—one regarding this escape from contact inhibition.

My colleagues, Dr. Connor and Dr. Marti, have reported recently on the effect of cortisone on cells of primary human amnion cultures. After these cells reach a state of contact inhibition of the proliferation, they are released from this state by treatment with cortisone and then proliferate further. Thus contact inhibition of normal cells can be obviated—as disclosed by Dr. Kruse—or broken as in the case with cortisone.

The second comment bears on the adeno-12-transformed cells that Dr. Yerganian spoke about. We have worked with these cells in our laboratory for several years, and we have always been amazed at the paradoxical discrepancy in the *in vivo* and *in vitro* systems. As few as 1–100 cells will produce tumors in the host of origin, the hamster, or in a heterologous host, the rat, if the hosts have been irradiated before transplantation. Yet we always have to explant or pass millions of cells *in vitro*—in fact, something like 1 million per ml—to get a decent monolayer. We could never work with low numbers *in vitro*.

By the way, I would like to confirm Dr. Yerganian's finding that, with cultivation *in vitro*, tumorigenicity and tumor antigens diminish in adenovirus tumor cells.

Dr. J. Furth: Would someone comment on contact inhibition and on the microenvironment of cells in relation to their growth *in vitro?*

Dr. D. J. Merchant: What I have to say is not concerned directly with the point that you raised, but it does have a bearing on this question of piling up of cells. I think that, aside from the phenomenon that Dr. Abercrombie and Dr. Ambrose described of contact inhibition which is a cessation of movement, the picture has been confused because, in fact, when cells reach confluency in monolayer in our experience, they have reached the plateau phase of the growth cycle for the particular medium in which they are growing. If one determines the cell numbers and relates them to the volume of medium in which the cells are growing, one finds that the cells stop growing the same way in a suspension culture in the same medium. They stop growing exactly at the same point.

In Dr. Kruse's experiments, he is renewing the medium in the same way that Dr. Earle showed a number of years ago—*i.e.*, if you centrifuge the cells in a suspension culture and give them fresh medium, they will grow up again, and you can keep doing this, so you can end up with populations of 10^7 or 10^8 cells per ml. But you have not achieved any greater production of cells per unit volume of medium expended.

I think we are dealing with a situation in which the cells have the ability to convert a certain volume of nutrient into protoplasm. There is a limit for each cell type, and they reach this limit in a monolayer just as they do in a suspension culture.

It just happens that this is confluency under the conditions that we usually grow cells. In Dr. Kruse's system, he is removing this barrier. This is probably the major reason the cells pile up.

Dr. J. Hanks: In science, we always run across some ghosts and goblins, such as contact inhibition or limitations of population densities.

The answer rests largely on what Dr. Merchant has said. But let me give you some examples from microbiology. If we have maximal population production in 10 ml of broth, we can solidify 9 ml of that system in agar. When we put the bacterial inoculum in the remaining 1 ml of liquid, we can grow the same number of cells that would grow in the 10 ml broth. The cells now are getting along very well at one log higher concentration than it has ever been possible to produce in the past.

This principle is demonstrated even more dramatically by placing the microbes in a cellophane-dialyzing bag and by having very large volumes of liquid medium on the outside or by permitting a slow flow of fresh medium to percolate through the pack of bacterial cells. In this way one can grow the cellophane bag solidly full of bacterial cells.

We shouldn't think of this phenomenon as being unusual. Merely think of the situation of the cell crowding in a rapidly growing embryo. The cells are packed side by side and stacked one upon the other. Obviously if one has a system for providing a continuous supply of nutrients and for eliminating end products, one may grow cells in actual solid packs.

One question asked, I think, by Dr. Kruse concerned the role of inhibitors or the removal of inhibitors. It has, in fact, been shown in some microbial systems that the volume of packed cells in a cellophane bag will be controlled by the volume of the external supply of medium. In a few instances, upon examination, it has been shown that the function of the big pool of medium, or of the continuous drip of medium, is definitely to remove water-soluble inhibitors.

Most of us know that in microbiology and in cell culture, advances have been made in recent years by using CO_2 incubators. This means that the gaseous atmosphere above a microbial culture or cell culture has become infinitely large. Among the mycobacteria, we know of examples where growth is inhibited by volatile end products of metabolism. If one keeps those volatile end products tightly impounded within screw-cap tubes, there is definite growth inhibition. If we place duplicate tubes with slightly loosened screw caps in a large container, the inhibition is relieved and there is a tremendous increase in growth rate and in the total cells obtained.

Thus, although tissue cells compete for living space on glass surfaces, such observations do not support the fallacious concept of growth limitation because of cell crowding.

Dr. Furth: In continuing the discussion, it would be proper to make a distinction between general growth promoters and so-called contact inhibitors. The substances of contact inhibition are said to be in the microenvironment of the cells, are elaborated by normal cells, and are deficient in the microenvironment of neoplastic cells. This problem is different from that of growing cells in large quantity.

Dr. Merchant: We shouldn't lose sight, because of what we have said here, of the fact that when you add SV40 virus or other transforming viruses to a culture and you see this piling up, you haven't changed the nutrient conditions. Therefore, you must have had an important change in the cell which enables that cell to utilize the nutrients quite differently than the cell did before it was transformed. Otherwise, you wouldn't get this piling up under these conditions.

Dr. V. Defendi: I believe there are three classes of substances that have been isolated or thought to be isolated from normal cells which inhibit growth of transformed cells.

One group is arginase, which we can forget about, I suppose. Another is "anomin," a substance, isolated by Dr. R. Bürk from nontransformed BHK 21 cells, which inhibits the growth of transformed cells. The third one is a cell membrane constituent described by Dr. Rubin in chicken-embryo fibroblast systems. Conceivably, the production of these substances could be essential for the regulation of the growth of normal cells in monolayer.

Dr. Todaro: Concerning the question of whether contact inhibition of cell division or the restriction to a monolayer that occurs under standard conditions in culture has anything to do with the neoplastic properties of this cell, I would like to briefly describe an experiment recently done by Dr. Robert Pollack at New York University. He took a polyoma-transformed cloned hamster cell, which made tumors very rapidly in the animal and grew well in culture, and tested whether he could select for a more contact-inhibited cell that might also have reduced tumor-producing capacity. He used 2′-deoxy-5-fluorouridine (FUDR) to kill all the cells that continued to divide after the monolayer was formed; in this way he selected for cells unable to grow past the monolayer. When this was done, starting from a cloned hamster tumor population, he got, after two cycles with FUDR a cell line that was almost as contact-inhibited as the normal cell and which when put back in the animal no longer produced tumors.

The hypothesis is that if contact inhibition has anything to do with tumorigenicity and if you select against cells that pile up when they become contacted, you should find that the cells you have selected in fact have lost tumorigenicity. This apparently is the case.

Dr. V. J. Evans: Dr. Kruse, have you done any wide spectrum of implantation studies with the cells you have grown in your perfusion system?

Dr. Kruse: No, Dr. Evans, we have not. I would like, in response to Dr. Todaro's statement, to ask Dr. Sanford, do you have a cell line that shows contact inhibition of movement, but is quite tumorigenic upon re-implantation?

Dr. K. Sanford: Most of the mammary carcinomas that we have grown in culture, I would call contact-inhibited. And they will grow as tumors. I would like to make an additional comment.

The first point may be a matter of semantics, a question of primary. I believe Dr. Evans has a primary culture in chemically defined medium that is neoplastic from normal tissue that has become neoplastic. And maybe the time in culture is more important than whether they are primary or secondary cultures.

I also want to call attention to the fact that in the early days, when embryo extract was used in the culture medium, it was quite easy to get multiple-layered cultures. So it must have something to do with nutrition as well.

I am not sure that I still believe there is such a phenomenon as contact inhibition. Many of these cells that require glass as a substrate when they have completely filled the flask cease to grow if they require glass substrate.

The open glass seems to be a stimulus to the cell to migrate and divide. It could be that the mechanism is not contact inhibition as such, but the lack of substrate of glass.

Many of the neoplastic cells seem to be able to grow in suspension. And the requirement for adhesion to glass may be an important factor.

I had one question. I wasn't quite sure how Dr. Todaro was using the term "transformation." Did he mean continuous immortality of the cells? I think he meant the morphological change.

The last point is text-figure 1 in the paper of Dr. Yerganian. I don't agree that the mouse and Syrian hamster cells show a limited lifetime. In our experience, these cells all will go on and grow indefinitely.

Dr. Todaro: By transformation, yes, we did mean this morphologic transformation, this focal pile-up of a colony derived from a single cell. These colonies when they are picked, though, do invariably contain the SV40 tumor antigen, have been shown by other people to contain SV40 messenger RNA, and can be cultivated indefinitely in a way that the normal-looking surrounding cells cannot.

Dr. G. Yerganian: Text-figure 1 was an adaptation of Green and Todaro's chart to illustrate retention of diploidy, not cell continuity.

Evidence for Oncogenic Properties of Heteroploid Cell Lines

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A heteroploid cell line has been defined as one in which more than 25% of the cells show an altered chromosomal constitution as compared with normal tissue cells of the species of origin (*1*). In early studies on the sequence of chromosome changes in normal tissue cells during culture (*2*, *3*), a pattern emerged. Initially, diploid or near-diploid cells predominated in the cultures with a shift toward a doubling or tetraploid level as cultivation continued. This shift presumably arose from failures in cytoplasmic cleavage or from chromosome doubling without nuclear division. In the resulting tetraploid cells, chromosomes were frequently gained or lost through mitotic abnormalities.

Also, extensive breakage of chromosomes and reunions occurred that led to structural alteration. Since the tetraploid cell contained a double amount of genetic material, this chromosome shuffling and rearrangement probably could be better tolerated than in a diploid cell; the resulting cells might also have some selective advantage *in vitro*. In any case, a heteroploid cell population finally evolved.

The heteroploid shift occurred early during the first few months *in vitro* in cells from certain species such as the mouse; in cells from man and other primates, the pig, rat, and to a lesser degree the Syrian hamster, the diploid state might be greatly prolonged [(*2*–*4*); for reviews *see* (*5*, *6*)]. These species differences were observed under a limited number of culture conditions and may possibly reflect inadequacies in our knowledge of the nutritional and other requirements of the cells.

The heteroploid shift has been thought to be directly related to neoplastic conversion of cells *in vitro*. However, since tumor cells showed a similar shift (*7*), the pattern appears to be associated more closely with the adaptation of cells to culture rather than to their neoplastic or normal state. As will be discussed later, there is no evidence at the present time that all heteroploid cell lines are tumorigenic or neoplastic.

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It was first observed in 1941 that normal mammalian tissue cells during culture *in vitro* may undergo neoplastic conversion (*8*, *9*). Such converted cells reimplanted into animals of the inbred strain of origin grew as invasive neoplasms that killed the host. Since no known carcinogenic chemical or virus was deliberately added to the cultures, these conversions of unknown cause have been referred to as "spontaneous."

In the past 26 years, there have been numerous reports from many laboratories of spontaneous neoplastic conversion in cell lines from a wide variety of tissues of different strains of several species of animals. Some of these have been tabulated (*10*).

In all reports, the cells had been implanted *in vivo* and grew as tumors. Most tumors arising from the tissue culture implants were sarcomas. Tumors from C3H mouse liver cells were an exception; they were diagnosed as anaplastic carcinoma resembling hepatoma.

As noted above, these conversions have been referred to as "spontaneous," which means proceeding without external stimulus. However, several observations from recent studies strongly suggest that these conversions are not "spontaneous" but may be induced by specific extrinsic factors.

In the past, at least two such extrinsic factors were postulated; *i.e.*, viruses and unidentified carcinogens in the complex biologic culture medium (*11*). Recent observations on the widespread occurrence of tumor viruses and the experimental induction of neoplastic conversion *in vitro* by certain tumor viruses (*12*, *13*) have suggested that viruses in the original tissues explanted or in serum-containing medium might be the cause. In our laboratory, we have explored this possibility further through a collaborative study (*14*); 27 murine cell lines derived from 12 different cell pools and grown in various media were examined with electron microscopy by Dr. W. T. Hall for morphologically detectable virus particles and by Dr. J. Hartley for the presence of complement-fixing (CF) mouse leukemia virus antigens and recoverable virus. An additional 19 lines from 8 different cell pools were examined for either virus particles or CF antigens. All 46 lines were also assayed in mice for neoplastic conversion. Of the 20 cell pools, 7 gave rise to lines showing evidence of leukemia virus contamination. Since most of these lines had also undergone neoplastic conversion *in vitro*, mouse leukemia virus cannot be excluded as possibly causing or influencing the neoplastic changes.

Of the remaining cell pools, 13 gave rise to lines showing no evidence of leukemia virus contamination. Many had undergone neoplastic conversion; others underwent conversion subsequent to tests. Eight cell lines also gave negative tests for the presence of 10 other murine viruses. These results suggest that neoplastic conversion can occur in the absence of leukemia and the other murine viruses detectable by the testing methods used. However, we are still looking for more sensitive test methods.

The development of cell lines in chemically defined culture medium without added serum, enzymes, or proteins is now making it possible to eliminate any unknown chemical carcinogens or hormones introduced from serum or other biologic products. Three murine cell lines developed by Dr. V. J. Evans and associates (*15*) and three by Dr. C. Waymouth (*16*) have undergone neoplastic conversion. However, several of these lines contained leukemia virus, and reports are not as yet available on the other cell lines. Mr. J. Jackson and I currently are assaying rat embryo cells initiated in chemically defined medium 3 years ago. No tumors have developed from the limited number of assays to date (*10*).

For the first time, a component(s) of the culture medium has been found to influence neoplastic conversion of cells *in vitro*. These findings stem from two observations by Dr. V. J. Evans and Mr. W. P. Andresen: 1) C3H mouse embryo or 3-day kidney cells grown in medium NCTC 135 + 10% horse serum undergo neoplastic conversion after a predictable period of culture *in vitro*, ranging from 98–188 days (*17*, *18*), and 2) if the horse serum is replaced by 10% fetal calf serum, the neoplastic conversion is significantly delayed or prevented (*19*). These initial observations have been extended by Dr. Evans and her group and have been confirmed and extended by the group working with me.

Two approaches have been used. 1) From one common pool of cells, paired lines have been cultured, one in horse serum and the other in fetal calf serum-supplemented medium NCTC 135. A total of 7 such pairs from 7 different pools have been studied. The test for neoplastic conversion was to implant the cells intraocularly into unconditioned mice or intramuscularly into irradiated C3H mice and to look for transplantable sarcomas from the implants. In all but one of the 7 series, the cell lines in fetal calf serum medium either failed to undergo neoplastic conversion or showed a significant delay over those grown in the horse serum medium. The one exception was the only line of those tested that contained type C particles and gave a positive test for CF mouse leukemia virus antigens. 2) Cell lines were initiated and grown in fetal calf serum medium; from these lines, cells were later transferred to horse serum-containing medium. From 4 different cell lines on fetal calf serum medium, 6 sublines were established on the horse serum medium, all of which became neoplastic before any evidence of neoplastic conversion in the parental lines.

Two of the lines on fetal calf serum medium still test as non-neoplastic after 2 years and a third after more than 3 years of continuous growth with subculturing at 1:2 or 1:4 splits every 7 days. These non-neoplastic cell lines by our tests, as studied by Mr. J. Jackson and Dr. R. Parshad and Dr. J. Mitchell in our laboratory (*20*, *21*), were heteroploid. These observations, as well as others in the literature (*22*, *23*), force us to modify the hypothesis that all heteroploid continuous cell lines can be considered neoplastic. In fact, the one cell line that still tests as non-neoplastic after more than 3 years *in vitro* has been heteroploid since the first 200 days in culture.

The cell lines were considered non-neoplastic because they failed to produce tumors under the same conditions of inoculum size, conditioning of host, and route of inoculation used for their parallel or derivative neoplastic lines in horse serum medium. The fetal calf serum appeared not to interfere with transplantability of the cells, since converted cells in horse serum medium when transferred to fetal calf serum medium grew as rapidly into tumors in mice as the cells maintained continuously in the horse serum medium. The cell lines could also be converted to neoplastic cell populations as noted above, so they had not lost their capacity to survive in the mouse. From studies by Dr. B. Barker and Dr. P. Farnes,[2] the converted cells in horse serum medium showed specific cytologic manifestations of neoplasia never seen in the nontumorigenic cells grown in fetal calf serum medium.

The observations on the effect of the serum suggest that some factor in horse serum absent or reduced in amount in fetal calf serum is carcinogenic when exposure is of sufficient duration. An alternative hypothesis is that some factor in fetal calf serum stabilizes the cells and thus prevents neoplastic change. Both of these hypotheses are being tested in our laboratory and the sera are being fractionated to identify the active components.

Since C3H mouse-embryo cells are probably the most unstable cells *in vitro* with respect to neoplastic change, the discovery of any factor that induces or prevents this conversion is an especially significant first step in developing reliable, non-neoplastic continuous cell lines for vaccine production.

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Evidence for Oncogenic Properties of Heteroploid Cell Lines—Discussion

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We believe that the schema of "cell senescence," postulating a sharply delimited lifespan for normal cell cultures, transgressible only if transformation is operated by an oncogenic virus, can hardly be accepted as a general rule for any tissue from any species at the present time.

Even for human cells, it does not seem to work always if we refer, for example, to data reported by Sandberg earlier in this Symposium.

In other species, maintenance in long-term cultures of predominantly euploid chromosome patterns were reported for rat cells by Krooth *et al.*, for Chinese hamster and Armenian hamster cells by Yerganian earlier in this Symposium, and by Macpherson for golden hamster cells, to quote only a few examples.

One can, of course, postulate fine unnoticeable changes in chromosomes accompanying this adaptation, but then it becomes a metaphysical debate.

Generally speaking, long-term cell cultures tend to have a specific pattern of evolution, characteristic for a given species. Concordant data indicate that in mouse cells chromosome numbers shift regularly toward hypotetraploid values, with appearance of some abnormal metacentric chromosomes between the 4th and the 10th month of cultivation. This is a very trivial fact at the present moment.

This pattern was observed first in our laboratory on cell lines developed from the lung tissue of C57BL adult mice. On the other hand, guinea-pig lung cells, cultivated repeatedly in our laboratory under the same conditions, developed fibroblast-like populations which remained grossly diploid for 25 generations in one case and for nearly 100 generations in another case.

Whether this difference between mice on the one hand and man or guinea pig on the other can be related to the fact that mice are thoroughly virus-contaminated and man and guinea pig may be virologically much "cleaner" remains an open question.

The C57BL cell lines we developed in our laboratory were, during the evolution to malignancy or nonmalignancy, virus-free as checked by electron microscopy.

When adaptation to an unlimited growth *in vitro*, accompanied by heteroploidy, occurs, the fundamental question is: What is the relationship between these changes and tumorigenic properties? Here, I am referring to what Dr. Sanford said already, adding some data concerning our C57BL cell lines.

We can state first that the appearance of tumorigenic properties is usually posterior to the heteroploid changes, and the capacity to produce tumors may appear only several months after all the cells become heteroploid.

Secondly, in some cases a transformed and heteroploid cell line may remain nontumorigenic after several years *in vitro*, as is the case for at least two of our C57BL lung cell lines that are being cultivated in calf serum, not fetal calf serum.

Similar data were reported from Dr. Sanford and Dr. Evans' laboratory and independently by Klein in Holland. Thus, the fact that there is no identity or strict equivalence between chromosomally altered *in vitro* "adapted" cells and *in vivo* invasive cells is demonstrated by the symmetric evidence that *a*) many "transformed" cell lines may be noninvasive *in vivo*, and *b*) many cancer cells are not growing as permanent cell strains *in vitro*.

In our hands, the occurrence of "transformed" but not invasive mouse cell lines was an unpredictable phenomenon. To explain it one can refer, among others, to the histological complexity of the primary lung tissue cultures.

In other words, besides the rules governing *in vitro* cell transformation according to species origin, other rules may exist, related to the tissue origin of the cultivated cells. And here the progress in the future can be assured only if we initiate cell lines by cloning primary cells at the beginning of the culture.

I wish to stress that invasiveness is obviously not an all-or-none phenomenon and the tumorigenic potential corresponds to a set of hereditary cell characteristics which must coincide in the transformed cell to make it cancerous. Furthermore, these characteristics, as Dr. Dawe pointed out, may evolve.

The peculiarity of cell culture *in vitro* is that there is no evident selective advantage in favor of more invasive cells and in some cases a decrease of tumorigenic potential *in vitro* can be observed—it can even be induced.

Finally, concerning the cell-to-cell transfer *in vitro* of tumorigenic properties, all our attempts to use subcellular material from spontaneously transformed heteroploid mouse cells regularly failed. Though, in nearly all cases in which cell hybridization occurred *in vitro* between tumorigenic and nontumorigenic cells, the hybrid cell descendants had invasive properties.

Evidence for Oncogenic Properties of Heteroploid Cell Lines—Discussion

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IT seems appropriate to a conference discussing the use of vaccines made in tissue culture to ask the question: What happens if an individual is inoculated with living human cells—either normal, transformed, or cancerous?

I will discuss the work done a number of years ago by Dr. Chester Southam and myself which involved the inoculation of such cells into two groups of human adult volunteers—patients with advanced cancer at James Ewing and Memorial Hospitals and normal adult males at the Ohio Penitentiary. Only the data from our first studies are presented here, since the same results were obtained in later studies which involved much larger numbers of volunteers.

Freshly grown tissue-cultured cells were trypsinized, washed, and counted, and as soon as possible thereafter, 1–4 million cells were inoculated subcutaneously into the forearms of the volunteers. The inoculation sites were examined daily for any local reaction and for the appearance of nodules.

In the early studies, all nodules were completely excised. In later studies, some nodules were allowed to regress. The results are shown in table 1 which gives the cell lines inoculated and the two groups of volunteers.

When human embryonic fibroblasts were inoculated, no tumors were produced in the 3 cancer patients or in the normal humans. We have many more individuals who received these cells and we have never seen tumors in any of them.

The transformed lines (now, I believe, being called converted)—were originally normal cells which through cultivation developed biologic or morphologic characteristics which made us consider them no longer normal—when inoculated into the cancer patients, specimens from 8 of the 11 sites biopsied showed living cells. In one biopsy site, a nodule recurred. These same cells given to the normal human volunteers produced no nodules.

Table 1.—Tissue culture cell lines inoculated into human volunteers*

Recipients	Cells inoculated				
	HEF†	Transformed		Cancer	
		Positive biopsy specimens	Recurrences	Positive biopsy specimens	Recurrences
Cancer patients...	0/3	8/11	1	11/12	4
Normal patients...	0/3	0/3	—	4/15	0

*Positive biopsy specimens/inoculated sites.
†Human embryonic fibroblasts.

A great difference between the two groups of volunteers was shown when the human epidermoid cancers were inoculated. In the cancer patients, there was very little, if any, local reaction. But 7–10 days later, a definite palpable nodule appeared. It was excised and showed growing cells in 11 of the 12 biopsy specimens. Also, at the biopsy site, there were 4 recurrences. In the later studies where the nodules were not removed, the HEp–3, our most tumorigenic cell line, produced nodules which persisted until the patient died of his own cancer. In one instance, we found a metastatic nodule in the axilla at autopsy 8 weeks post inoculation. In other words, some cancer cells inoculated into patients with far-advanced cancer were capable of sustained growth.

The picture was quite different in the normal humans. At the inoculation site, there was a definite reaction consisting of swelling, redness, and in some instances tenderness lasting sometimes as long as 2 weeks. Nodules also appeared, although they were difficult to define due to the local reaction. However, in 4 of the 15 biopsies performed, viable tumor cells were found. There were no recurrences. Under ordinary circumstances, the tumors regressed spontaneously within 2 weeks. Even when the individuals received the most tumorigenic line, the HEp–3, the nodules disappeared in 4–6 weeks.

I mention this rather ancient history to refresh your memory and also to point out that there are two things governing whether cells will produce tumors in individuals. One is the kind of cells inoculated and the other is the recipient. In other words, if a normal human is inoculated with cancer cells, we wouldn't expect him to develop a tumor, whereas in a patient with far-advanced cancer, a growing tumor could possibly be produced which would persist. Even with the most tumorigenic cell line, inoculated into the most susceptible patients, 1 million cells were necessary to produce a nodule. In other words, cancer cells grown under ordinary tissue culture conditions are probably less dangerous in a vaccine than the unseen microorganism, either bacterial or viral, that they may carry.

GENERAL DISCUSSION

Dr. J. Furth: Dr. Moore introduced a chapter which hasn't been touched on yet, but which is so vital in oncology—the immunological surveillance, which operates not only versus homologous but also versus autologous cancer cells. A transformed neoplastic cell may grow *in vitro*, but may not be re-graftable in the host of origin if it is modified immunologically.

Dr. Orr (NIH): Dr. Sanford, in view of your evidence incriminating horse serum and cell conversion, what has been the experience with growing the WI–38 cells in horse serum? Or can anyone else answer this question?

Dr. K. Sanford: I don't know of any work on growth of WI–38 cells in horse serum. However, a neoplastic conversion in human cells would be difficult to detect with any reliability.

Dr. L. Hayflick: Most cultures of WI–38 that we and others have grown in the past 5 or 6 years have all been grown in calf serum. On the one or two occasions that we tried horse serum, we did not see any transformation of the type described here.

Dr. Sanford: Several lines in our laboratory have been grown on horse serum, fetal calf serum, or calf serum. With cells derived from a common pool, the ones on calf serum underwent neoplastic change almost as readily as those on the horse serum medium. Those grown on fetal calf serum showed the delay.

Dr. B. A. Rubin: Dr. Sanford, we, of course, in growing lots of diploid cells are very interested in your work.

Have you heat-inactivated any of these sera and, if so, what happens? You say you are fractionating these sera, but there is a very simple question that has come up, for instance, in regard to fetal calf serum about whether there are labile metabolites or something like that which might be affected. We have heard a lot about the possibility of viruses in sera in the last couple of days. So this is an important question. What is the effect of heating?

Dr. Sanford: We have not tried heated serum.

Dr. Furth: Dr. Sanford, to answer some of the problems raised, symmetrical systems are needed. The commercial horse sera come from adult or aged animals; calf and fetal calf sera are from another species, taken at diffrent ages. For basic comparison, it would be desirable to use sera of different species at various periods of life.

Dr. Sanford: Dr. Evans and Dr. Mitchell are exploring the effect of young and old, fetal and adult sera.

Dr. Furth: The old would be very interesting because there is ample evidence of transformation in old cells. They are innumerable and compatible with life.

Dr. K. G. Brand: Dr. Moore, did you ever extend your work to persons with diseases other than cancer, especially those with certain immune deficiencies? Also, could age be a factor, because your normal volunteers probably were young people in contrast to the older cancer patients?

Dr. A. E. Moore: The same type studies have been done on a group of debilitated volunteers who were in comparable condition to the patients with far-advanced cancer. They reacted more like the normal individuals. Regarding your second question about the age distribution, in the Ohio Penitentiary, many inmates come back repeatedly so that we did have quite a number of older individuals. Although I don't have the exact figures, I don't think age is a factor.

Dr. Furth: Did you say something about the immunological competence in age? Undoubtedly even the lymphocyte system deteriorates with age.

Dr. Moore: This study was continued with Dr. Southam to study the immunological resistance developed to these transplants when the individual received repeated inoculations of the same type of tissue cultures. Other than that, I don't think a study has been done on the actual immunological competence.

Dr. Furth: And neoplasia?

Dr. Moore: The general feeling is that the individual with cancer develops more tumors and more progressively growing tumors than the normal individual.

Dr. Furth: How do cancer patients respond to a standard antigen at various stages of the disease compared to normal persons and persons with diseases other than cancer?

Dr. Moore: We know that cancer patients are perfectly capable of developing antibodies from previous virus studies. They develop them just as well as a normal individual.

Neoplastic Properties of Animal Cell Lines—General Comments

Ian Macpherson,[1] *Medical Research Council, Experimental Virus Research Unit, Institute of Virology, University of Glasgow, Glasgow, W. 1, Scotland*

We have had some discussion on the correlation between heteroploidy and tumorigenicity in cultured cells and examples have been given of heteroploid cells that do not induce tumors. Despite these observations we should not lose sight of the fact that a positive correlation exists between heteroploidy and tumorigenicity in cultured cell lines.

The use of such cell lines in the preparation of human viral vaccines presents an unknown hazard. This does not come from the cells *per se*, since the elimination of viable cells from a vaccine presents no great difficulties, and even in the unlikely event of a few viable cells surviving they probably would not cause a tumor in an immunologically mature host.

The risk in the use of heteroploid cells is the possibility that their neoplastic properties were induced by a subcellular entity which could persist in the cells and remain active in the vaccine. We must weigh this hazard against any advantage gained from the use of these cells in vaccine production. At present there are no instances in which the advantages are sufficiently striking to justify the risk. However, this situation may change and the scales may not always be so heavily weighted against the heteroploid cell.

The most desirable goal in virus vaccine production is the preparation of highly purified virus or viral protein. Earlier in this Symposium Dr. Bachrach described how this could be achieved with the virus of foot-and-mouth disease grown in the BHK21 line of hamster cells. However, the large-scale production of virus requires the large-scale production of cells, and the most economical method of growing large quantities of cells is in suspension, especially under steady-state conditions in continuous culture. Such methods are now practical but are only applicable to heteroploid cells. It is therefore important to discover as much as possible about the nature of spontaneous transformation in different cells so as to be able to assess whether a purified virus vaccine could be safely prepared in such cells.

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Our knowledge of spontaneous transformation is meager and almost entirely descriptive. We know that the cells of certain species have characteristic probabilities of becoming spontaneously transformed; *e.g.*, normal human cells and avian cells consistently fail to undergo spontaneous transformation, whereas mouse cells and monkey cells frequently give rise to stable cell lines. These differences in behavior may be due to: 1) different probabilities that mutation leads to autonomous growth of the cells; 2) variation in the frequency with which the cells of particular species carry transforming agents with them into culture; or 3) differences in the way the cells react to transforming agents added to the cultures either in the media or from the operators.

The type of agent most likely to cause transformation of cells is an oncogenic virus. *Mycoplasma* should also be considered, especially since we know that they are capable of causing morphological changes in BHK21 cells and conferring on the cells the ability to grow in agar suspension culture (*1*). I was interested in Dr. Sanford's observation that mouse cells frequently transform when grown in horse serum medium, but much less frequently when grown in medium containing fetal calf serum. These results recall the observation of Shepard and Lunceford (*2*), that the T strains of *Mycoplasma* grow very much better in horse serum medium because it has a higher content of urea than the sera from other species. It would be interesting to find out if T strains of *Mycoplasma* are involved in the transformation of mouse cells.

If it can be shown that heteroploid cell lines result from the action of specific agents which can be eliminated during virus purification, we may then reconsider the use of these cells for the preparation of vaccines and antigens.

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Neoplastic Properties of Animal Cell Lines—General Comments

Robert E. Stevenson, *Union Carbide Corporation, Tarrytown, New York 10591*

As Dr. Sanford stated earlier, there is no evidence at the present time that all heteroploid cell lines are neoplastic. In fact, data presented by Gartler and confirmed here by members of the Cell Culture Collection Committee would indicate that many human heteroploid cell populations thought to be exemplary of a high frequency of spontaneous conversion represent in all probability extrinsic contamination with the HeLa cell line.

We should not lose sight of the fact, however, that proven heteroploid and neoplastic conversion can and does occur with varying degrees of probability following certain environmental stimuli applied to cells. Viruses, radiation, and carcinogenic chemicals are well-known examples of such stimuli, and viruses have been of particular interest, since the converted state they engender is reproducibly tagged with one or more consistent markers.

Radiation- and chemical-induced conversions do not share this convenience in manipulation, and the painstaking work by Prehn and others (*1*, *2*) over many years has shown the practical problems encountered in such studies. More recent work by Sachs and coworkers (*3*) with contact inhibition of mitosis shows promise, however, that a practical means may be at hand for determining the practical limits of "antigenic" variation between clones of heteroploid cells derived from a stemline by different events of a similar stimulus.

Other ecological factors in cell culture systems, largely ignored to date, were touched upon by Dr. Hanks earlier in this Symposium in his speculations about medium composition and its effect on cell modulations.

Precise work by Nelson-Rees (*4*) showing the profound effects of heterologous serum on the karyotype of primary cultured cells makes one wonder if heterospecific or even homologous antibodies might not also be villians in this set piece.

What of the mixture of enzymes, bacterial cells, and detritus called trypsin which we blithely employ to pry cells loose from their resting places?

What of the effects of pendulum swings of aerobic to anaerobic conditions and fluctuations of CO_2 concentrations? These have not been well studied as factors in heteroploid conversion pressures under controlled conditions.

Dr. Evans and Dr. Sanford plan to look for a protective substance in fetal calf serum which will retard conversion. Perhaps what they are seeking will not be there, because the stimulus comes from something like a heterospecific antibody versus mouse cells which is present in horse serum but lacking in fetal calf serum.

Time will tell, and one has to start with some hypothesis to test. One thing is clear as a result of these meetings, however: A new era of *cell ecology* is required. No longer can we afford to ignore or dismiss the effects of the other parameters of *in vitro* culture on the net results and confine our attention only to the nature of viral agents used for the inoculation of the substrate.

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Neoplastic Properties of Animal Cell Lines—General Comments

Helene W. Toolan, *Director*, *Putnam Memorial Hospital*, *Institute for Medical Research*, *Bennington*, *Vermont 05201*

PERHAPS in a conference of this nature, the best service I can render is an account of some of my experiences with heterotransplantation that are pertinent to the problems we have been discussing. In the short time available, three things may be noted:

1) In our experience, heteroploidy is not necessarily associated with the malignant state. One of our human tumors; *e.g.*, an embryonal rhabdomyosarcoma now continuously transplanted for over 14 years in rats conditioned by X irradiation and cortisone, has maintained a normal male karyotype of 46 chromosomes during that whole period. It was last examined this spring by Dr. Charles Miles of the University of Utah who has examined it repeatedly before.

In general, we can say that the human tumors we have maintained in small animal hosts appear to be more stable as far as karyotype is concerned than tissue cell cultures derived from them—a matter for some thought.

2) We have been impressed by the pleomorphism of one of our transplanted tumors—particularly when it is in a stress situation. H.S. #1, a very slow-growing tumor in its human donor, was first thought to be a metastatic nodule of a clear cell tumor of the kidney, due to its vacuolated "foamy" cytoplasm. When first implanted into conditioned rats, however, it grew to an enormous size within 14 days and killed each of its heterologous hosts. We interpreted this finding as indicating that the neoplasm was under some restraint in the original host and only realized its full potential in the defenseless rats. It is noteworthy that the fast-growing tumor looked like a fibrosarcoma composed of uniform elongated cells. After 15 years of transplantation, H.S. #1 continues to look either like a fibrosarcoma or an undifferentiated sarcoma. Yet whenever this tumor is put into improperly and/or insufficiently X-irradiated hosts—falls upon hard times as it were—it reverts to its original appearance of epitheloid-like vacuolated cells, indistinguishable morphologically from the original tu-

mor. This is an example of the cell appearance altering in relation to the resistance offered by the host.

3) We would like especially to caution those investigators who attempt to assay the malignancy of a tissue culture entirely on its ability to proliferate or form nodules in the conditioned, heterologous host. They should be well aware that many normal and embryonic cells are often more consistent in their capacity to grow in a homologous or heterologous host than the proven metastatic, highly malignant tumor. For example, tumor cells with many secreting and/or autolytic enzyme components; *e.g.*, those derived from gastric and intestinal tracts, are very difficult to establish as transplantable tumors. Electron microscope studies indicate that they disintegrate before they can survive the vicissitudes of a transplantation procedure.

On the other hand, embryonic lung, skin, and kidney and undifferentiated gut and stomach can be depended on to transplant well in about 100% of the attempts made. Even normal tissues such as skin and thyroid do well when transplanted. Tissues of aged individuals often show remarkable "rejuvenation," in fact, when placed in the young animals.

I will present a few figures to illustrate these points.

Figure 1 shows the H.S. #1, of which I spoke, where the original neoplasm looked like a clear cell tumor of the kidney; it can revert to that type, but under ordinary circumstances looks like a fibrosarcoma. It is a figure a number of you have seen before and it indicates the potentialities for growth of transplantable human tumors. In this case the H.S. #1 is growing in a cortisone-treated hamster.

Figure 2 shows the same tumor in an X-irradiated rat. This picture was taken 12 days after implantation and shows the growth that can be obtained. In fact, we have estimated that about four carloads of this tumor have now been grown over the years in animals, although the original human donor is still alive and well.

Figure 3 illustrates normal thyroid tissue from an 83-year-old man. This tissue had been implanted 15 days before in an X-irradiated rat. The original material was practically atrophic. Yet, when placed in young animals, either conditioned rats or hamsters, it promptly plumped up and looked like a normal thyroid with many mitotic cells present.

Figure 4 shows an embryonic human lung at 3–4 months of gestation. Only a small amount of differentiation is present.

Figure 5 shows the same lung after 10 days' growth in a conditioned hamster.

Figure 6 shows a "whole" lung which was grown from a homogenate of human embryonic lung tissue implanted in the hamster cheek pouch. It remained for 40 days in the hamster pouch, at which time it was removed. It grew progressively during this whole period.

Again, I would like to emphasize that it is next to impossible to transplant these normal or embryonic tissues, in contrast to tissues of neoplastic origin.

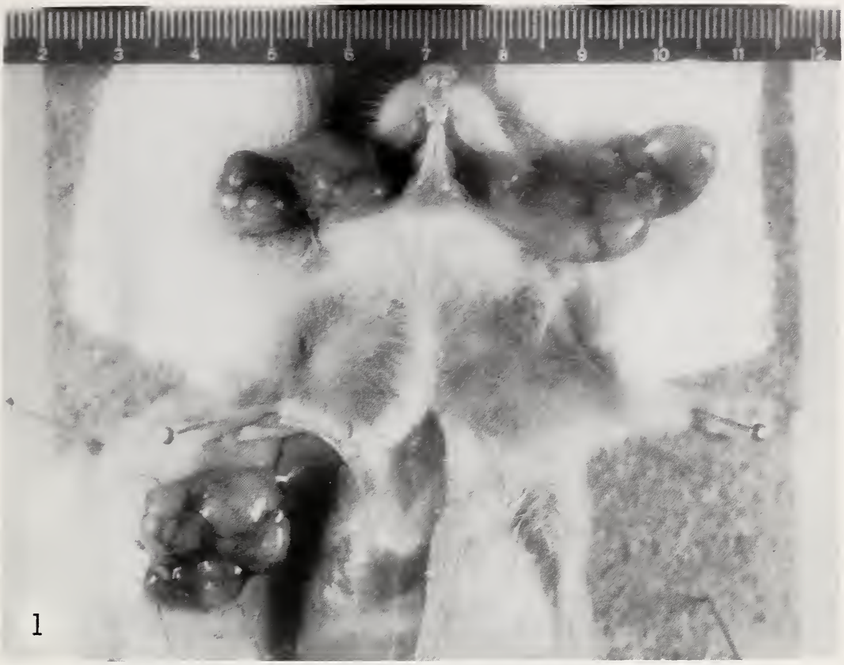
Figure 7 shows an undifferentiated rat gut. I would like you to see some of the capacities that it can display.

Whether one uses the proximal end of the embryonic gut (fig. 8) or the distal end (fig. 9), it is amazing what differentiation and development of embryonic material can be obtained in the conditioned host. All of these embryonic materials were placed as a brei on the chest wall of the recipient.

Embryonic stomach can also develop into fully functioning cells (fig. 10).

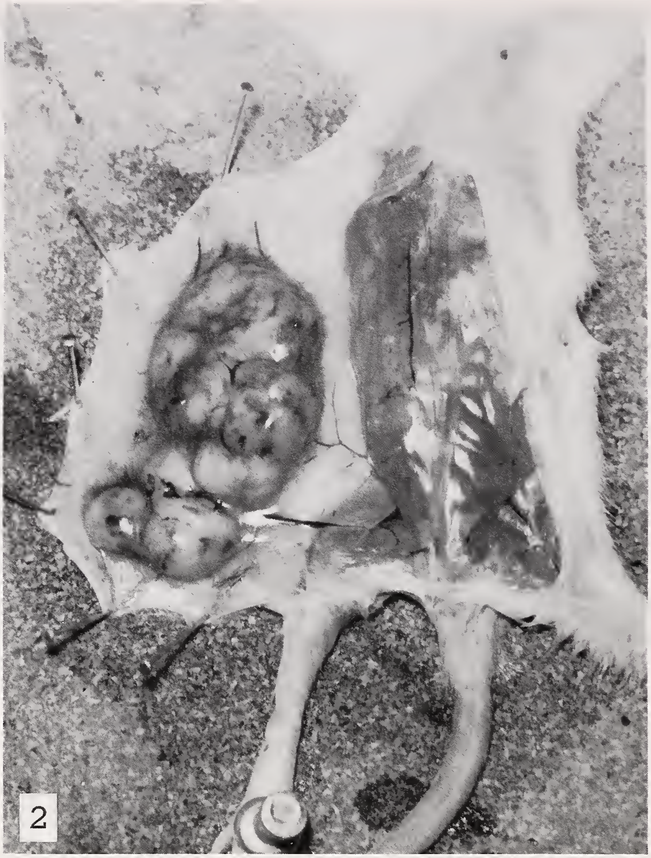
Finally, figure 11 shows you what originally was embryonic stomach, but which is now differentiated and growing on the chest wall of a conditioned rat. It is functioning very well; indeed it is secreting and provides an excellent surface on which to do a number of experiments.

In summary, the transplantability of each cell type must be considered in the light of its own metabolism requirements and its own constitutional makeup, not just on the basis of whether it is presumably normal or malignant.



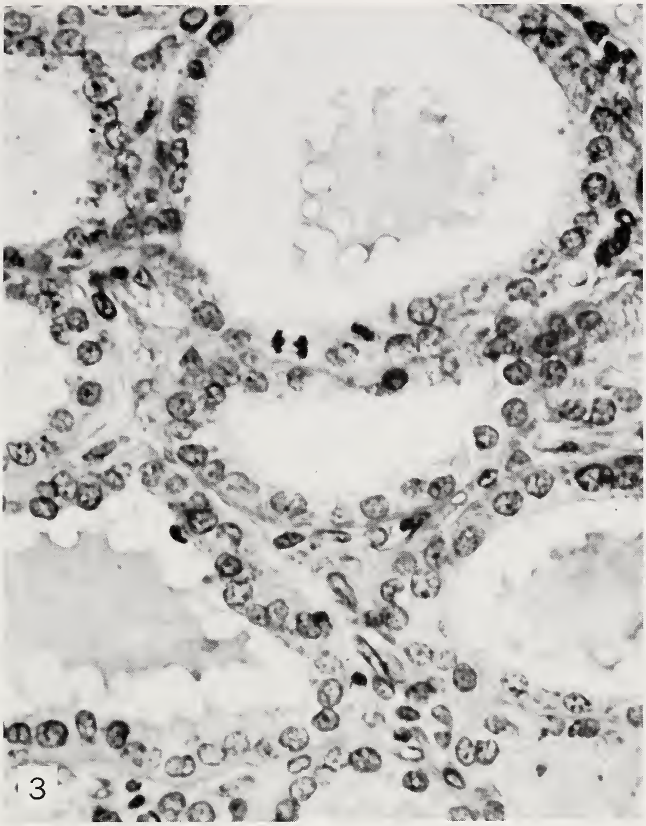
Annals of the New York Academy of Sciences 59 : 394. 1955.

FIGURE 1.—H.S. #1 tumor growing in a cortisone-treated hamster.



Medical Clinics of North America 40 : 1, 1956.

FIGURE 2.—H.S. #1 in X-irradiated rat.



Cancer Research 17 : 248, 1957.

Figure 3.—Human thyroid from 83-year-old male. Thyroid is growing in X-irradiated rat.

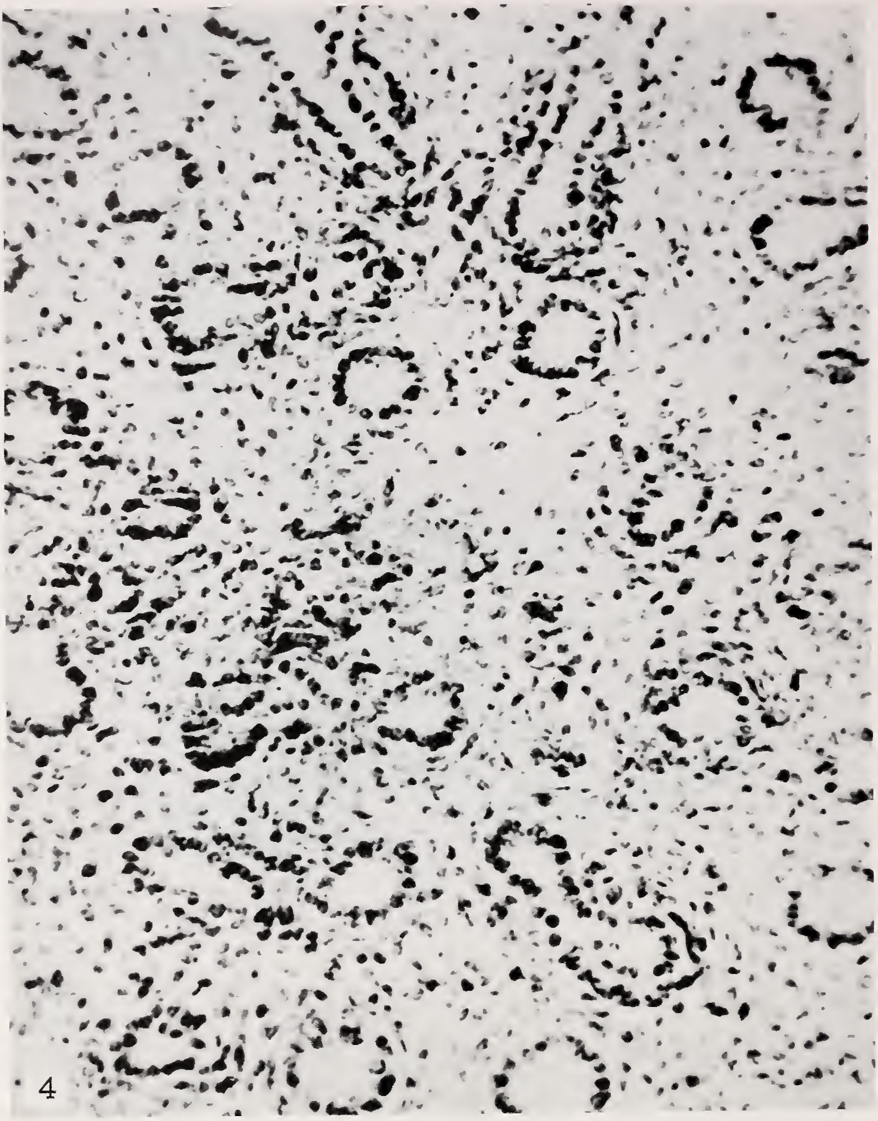


FIGURE 4.—Original human embryonic lung (3–4 months' gestation).

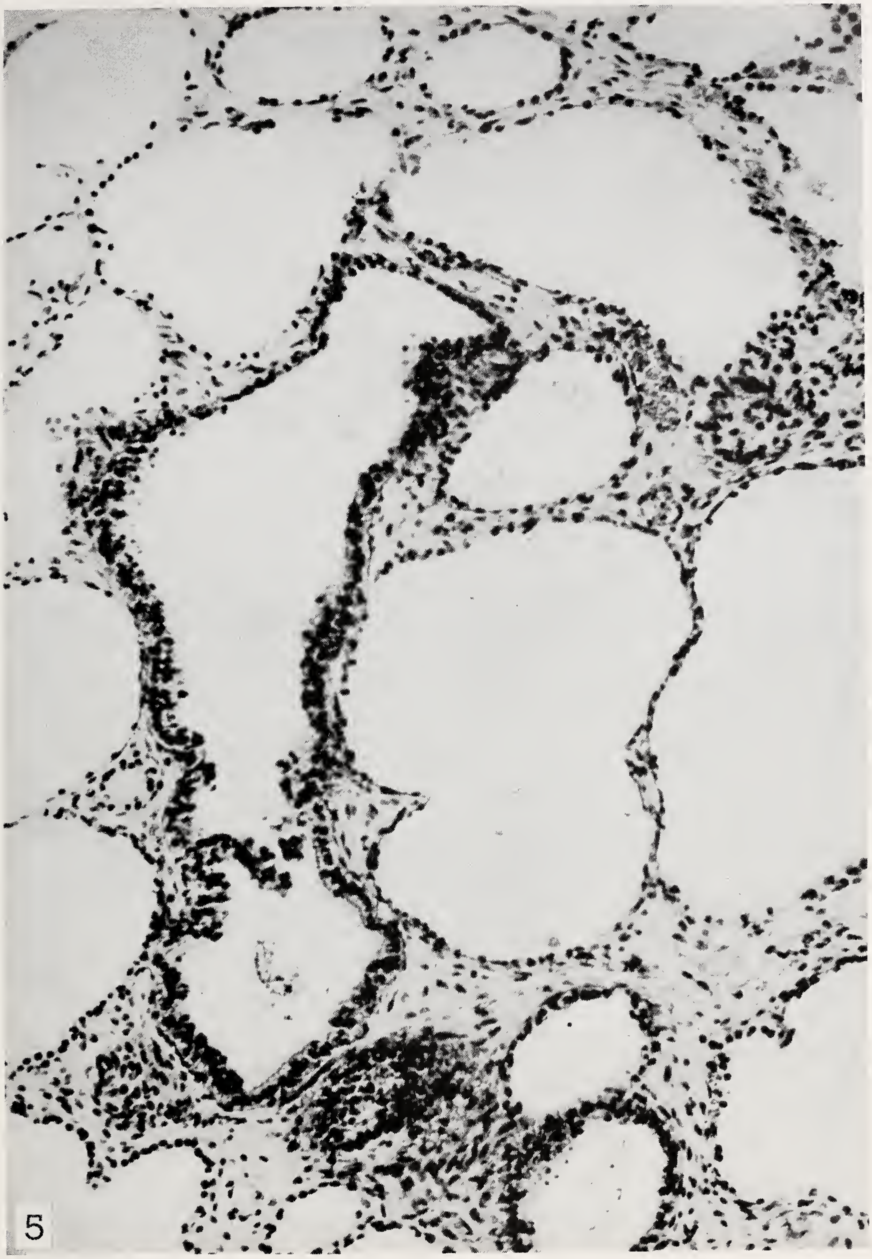


FIGURE 5.—Same lung as shown in figure 4 after 10 days in a conditioned hamster.

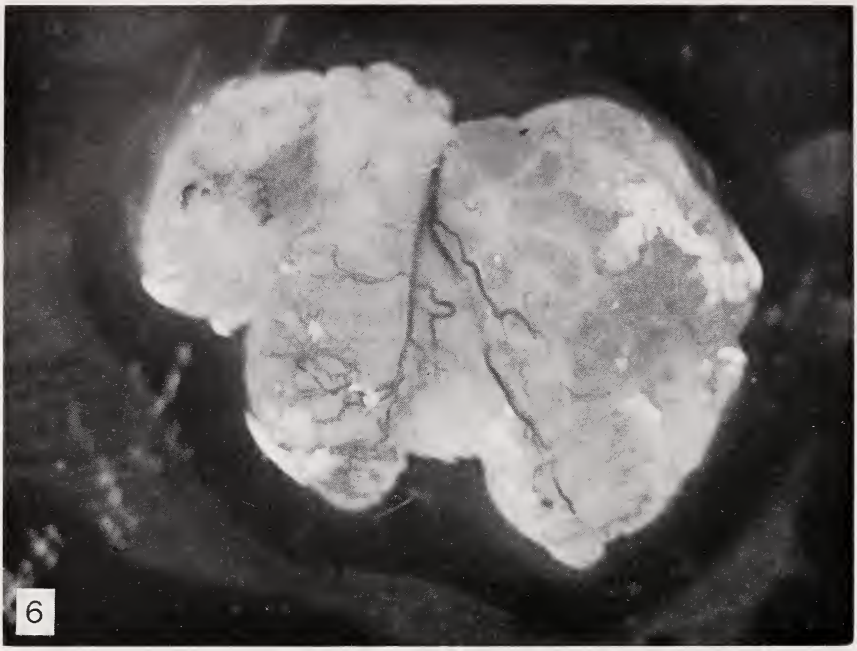


Figure 6.—"Whole" human lung in a conditioned hamster. Originally it was derived from a mince of human embryonic lung of 3–4 months' gestation. It was removed after 40 days in the hamster pouch.

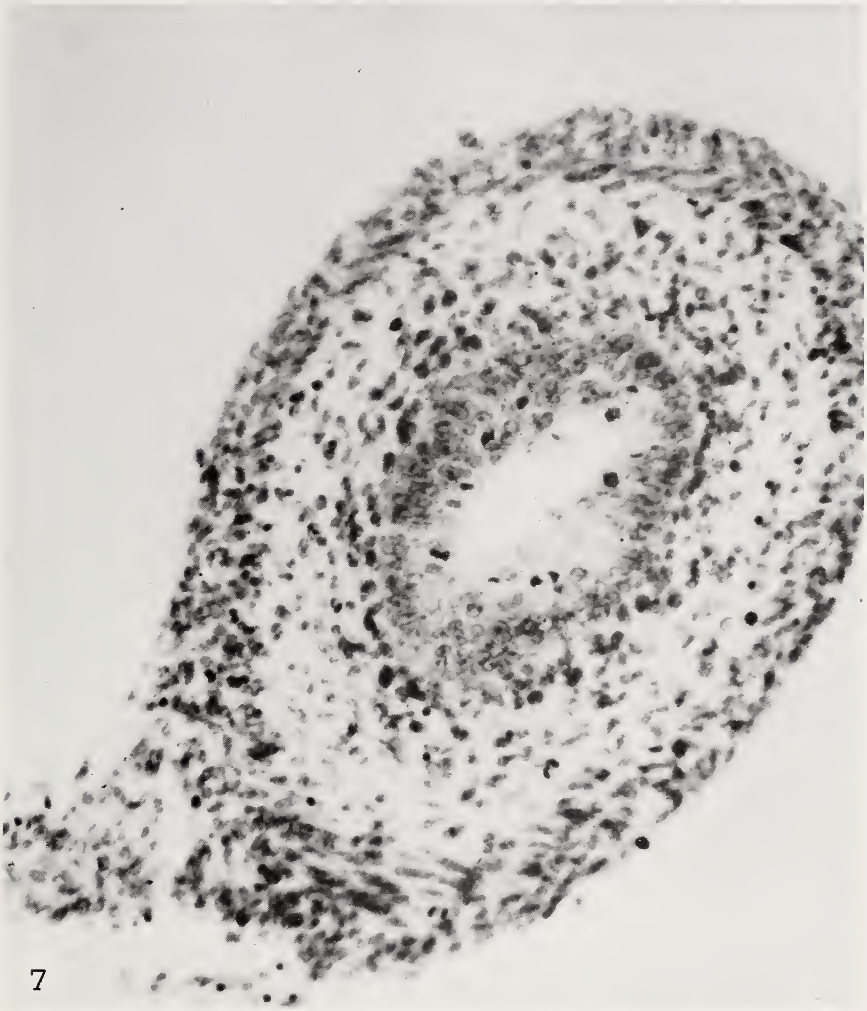
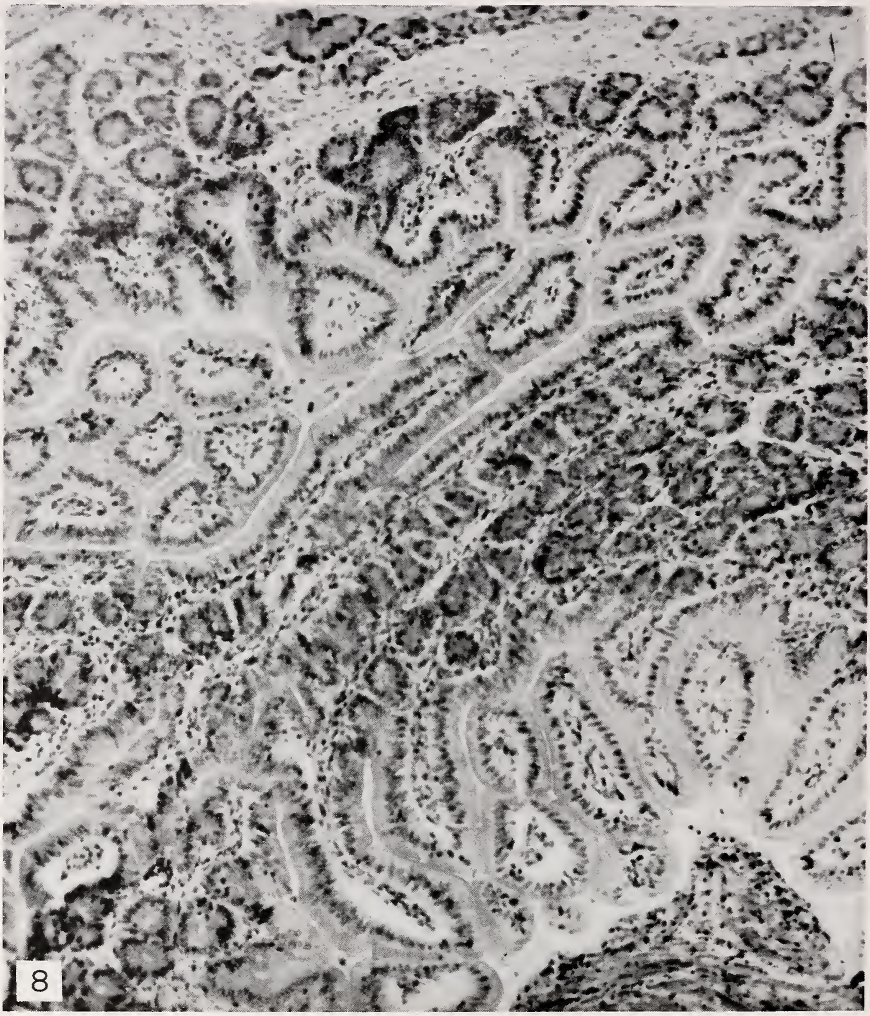
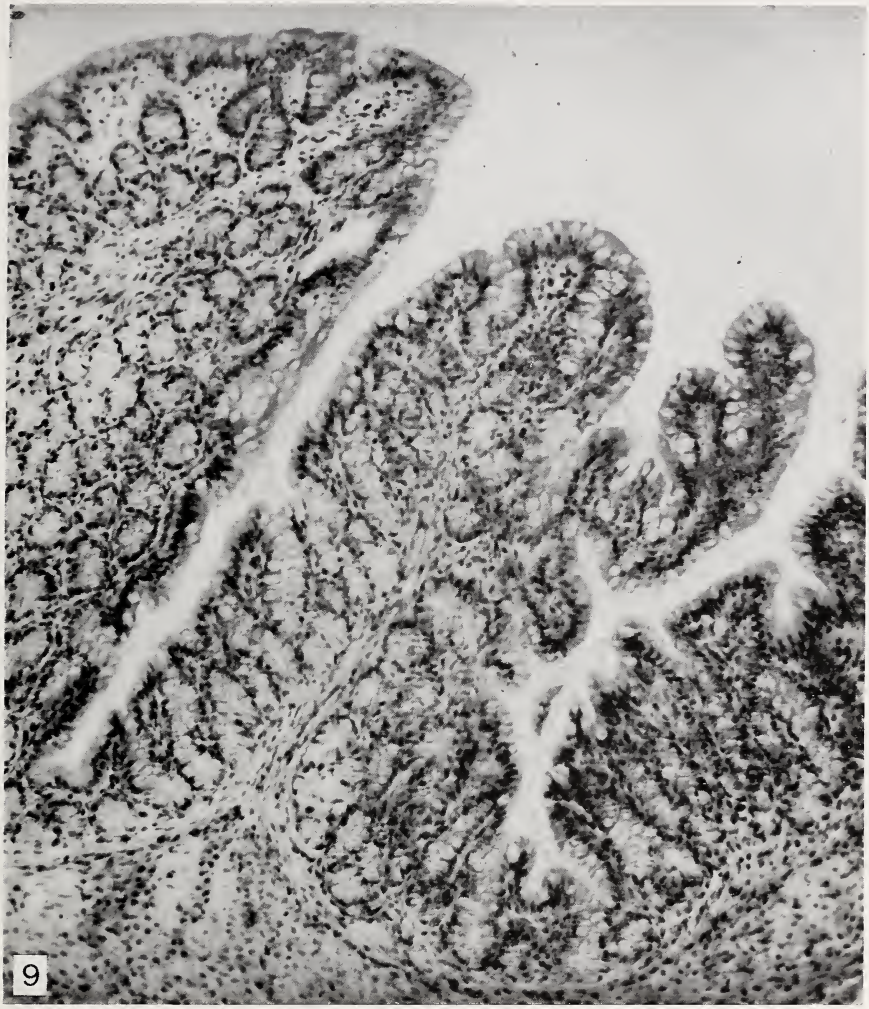


FIGURE 7.—Original undifferentiated embryonic rat gut.



Cancer Research 17 : 707, 1957.

FIGURE 8.—Growth of proximal area of gut shown in figure 7 on a conditioned rat 30 days after being placed as a mince on the chest wall.



Cancer Research 17 : 707, 1957.

FIGURE 9.—Growth of distal area of gut shown in figure 7 on conditioned rat.

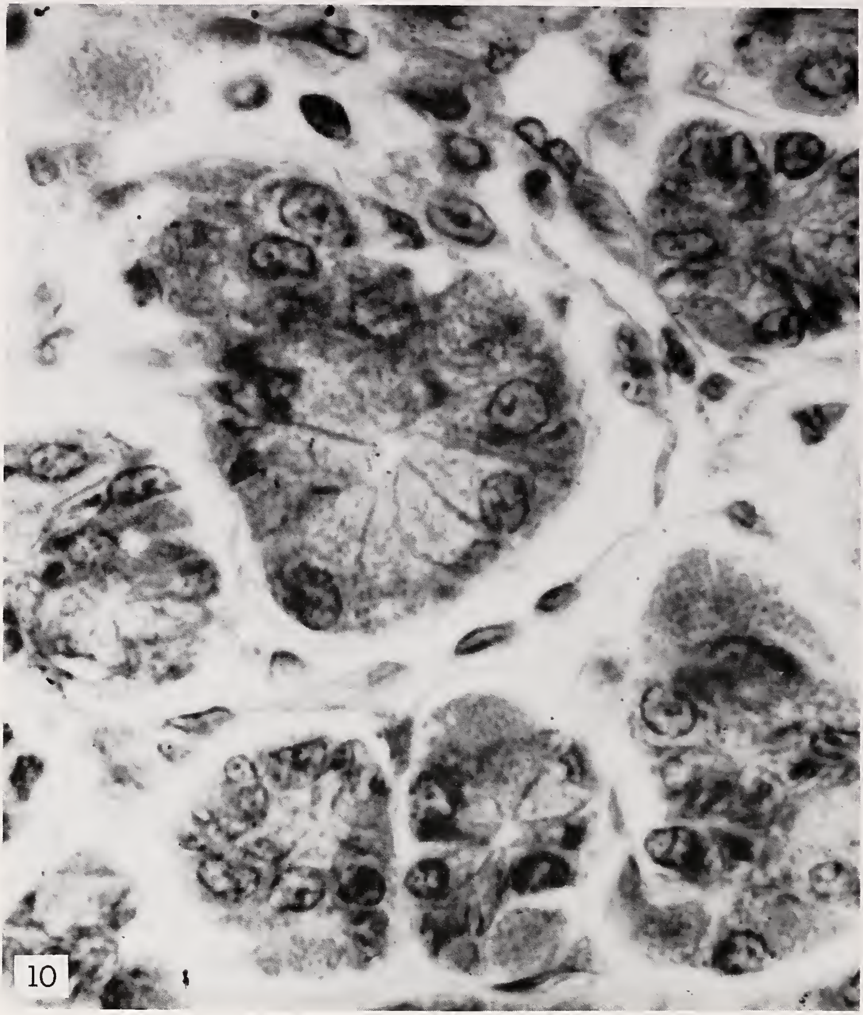


FIGURE 10.—Development of rat embryonic stomach cells into mature elements on the chest of an X-irradiated rat.

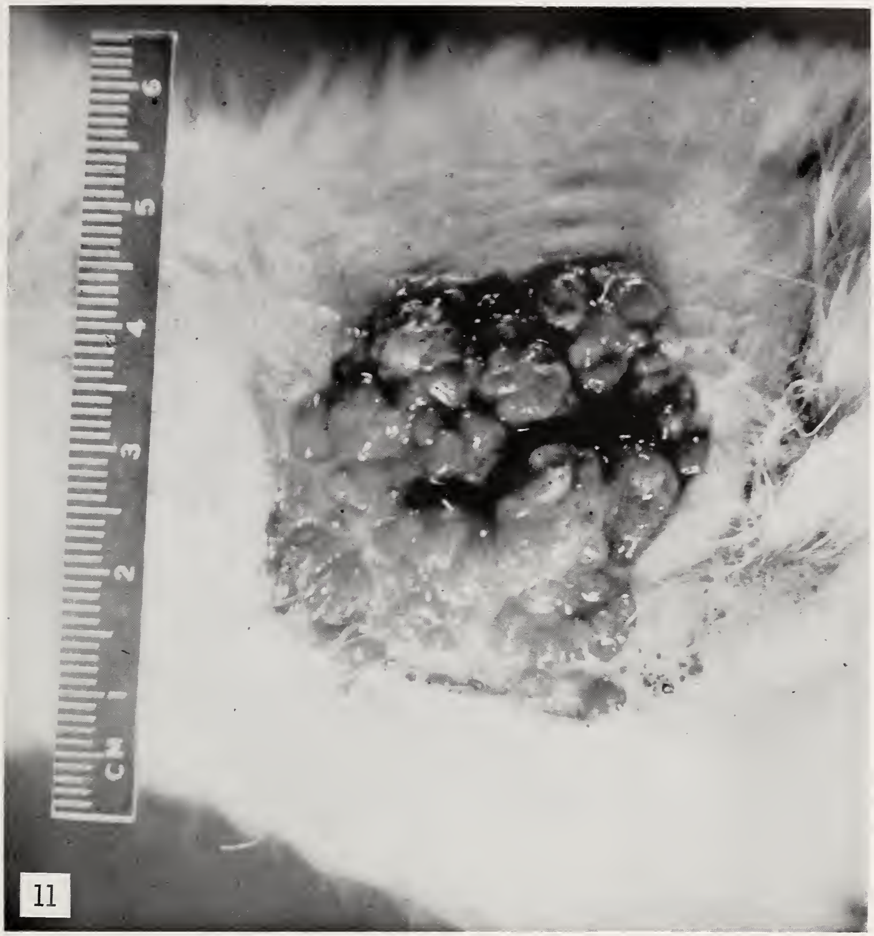


Figure 11.—Embryonic rat stomach mucosa growing on the chest wall of an X-irradiated rat.

GENERAL DISCUSSION

Mrs. L. C. Minecci: I would like to return to a point discussed earlier and ask that a distinction be made between cell layering and the "piling up" of cells. Normal diploid fibroblast cultures, such as WI–38, which we usually refer to as "monolayer cultures," are not in fact always single layers of cells, but are often orderly *multilayered* cultures. This is quite evident on examination of the cell sheet, in which the long axes of the fibroblasts may have different polarities in the several layers. I am certain that this phenomenon is not the same as the loss of contact inhibition which was referred to earlier as an indication of cell oncogenicity.

Dr. L. Hayflick: Mrs. Minecci's statement is substantially correct and actually something that I wanted to refer to earlier this morning, but there was insufficient time for me to be recognized.

The normal human diploid cells, as Dr. Moorhead and I have described originally, do pile up two or three cell layers thick. There is photographic documentation of this in the original publication. Certainly, this does not occur to the degree Dr. Kruse described in more elegant situations of perfused or nonstagnant tissue culture.

We accomplish multilayering merely by adjusting the *p*H at the time of cell confluency. The cells then continue to proliferate and lay down collagen and fibrin between the layers as was described in the original report in 1961. This implies that the requirement for a glass substrate or the need for fresh nutrients, as had been surmised, is not required for the continued piling up of normal human cells *in vitro*. If piling up is at all related to malignancy, it is not a question of whether cells pile up, but how they pile up—that is, whether the process is orderly or disorganized. Normal human cells pile up in a very orderly manner.

Dr. D. Franks: I would like to ask Dr. Toolan a question. I am afraid I am not very familiar with this work, but is there any evidence about the contribution that the host makes to these organized structures and the contribution that the donor material makes to it?

Dr. H. W. Toolan: In our particular work cited today, except for the one instance where several animals purposely were not properly irradiated, the hosts are nullified and are defenseless.

Dr. Franks: But the point is whether host cells joined in the formation of these structures. Perhaps something from the donor is put in which makes a substrate on which host tissue can build up these structures.

Dr. Toolan: It has been well known since the early 1900's that the host supplies the stroma. The human tissues that we see are parenchymal cells.

Dr. Franks: But have you made any specific tests by looking for marker antigens or using chromosome markers to see whether you can identify positively host cells in these structures.

Dr. Toolan: These transplantable human tumors have been analyzed by Dr. L. Korngold for human tumor antigens using the Ouchterlony technique and by Dr. Levan and Dr. Miles for their karyotype. They were shown by both methods to be human.

Dr. M. de Oca (Division of Biologics Standards): A question to Dr. Toolan. Do you find metastases in the case of tumors transplanted in hamsters?

Dr. Toolan: Only one of our transplantable human tumors metastasizes, possibly since they are in the conditioned host for a relatively short time. That is HEp–3 which Dr. Moore spoke about this morning. It metastasizes very readily.

If HEp–3 is put into the egg and the chick manages to hatch and survive, it will have metastatic tumors in practically every organ examined.

The other transplantable human tumors do not metastasize. I think that in general this is true of most murine tumors as well, is it not, Dr. Furth?

Dr. J. Furth: True; but if given time, and the tumors are resected or restrained (as human tumors), they spread more often than generally believed.

I should like to close with two general comments. To explain "spontaneous" carcinogenic transformation in tissue cultures, the trend is to postulate the presence of a virus or other carcinogens in the medium. The "miracle" is that the DNA, this giant molecule, usually copies itself perfectly. Any subtle error may result in change of the genome or of its expression, some of which can lead to a defect of its growth control. The theory of replication errors leading to spontaneous mutations seems as well conceived as mutagenesis by carcinogens.

Now in tissue cultures, the chances of replication errors are greatly increased merely by the enhanced replication of cells.

A second comment: There should be a better understanding of the character of "transformations"; irreversible vs. reversible; differentiation vs. gene mutation. The common irreversible transformations in tissue cultures are probably due to gene mutations or abnormal differentiations. Neoplastic transformations which go with unrestrained growth in normal hosts appear to be rare. Potentially neoplastic transformation of cells with no growth, due to immunologic modification, is a theoretical possibility, demonstrable by analysis of clones of cells altered *in vitro*. *In vivo*, they are "dead-born."

Session IV

Viral Oncogenicity and Viral Vaccines

Chairman: Karl Habel
Rapporteur: William Murphy, Jr.

Documentation and Classification of Oncogenic Viruses

Robert J. Huebner, *Chief, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases,*[1] *Bethesda, Maryland 20014*

THE established oncogenic viruses fall into four broad groups of viruses, three of them DNA and an RNA or myxovirus group:

Oncogenic groups of viruses
DNA
Poxviruses
Papovaviruses
Adenoviruses
RNA
Avian leukosis-sarcoma viruses
Murine leukemia-sarcoma viruses

There are those who feel that any virus can cause cancer in certain circumstances; others feel that when a virus causes cancer there is nothing very specific about it. But the fact is that the viruses that do cause cancer have a pattern about them and it is now clear their oncogenic activity is a virus gene-directed phenomenon.

It is interesting that not only do a number of different representatives of each virus group cause specific types of cancer in the same species—and this really I think is a very important point—but with every one of the groups we have listed here there is not just one representative that causes tumors in the respective species, but a number of them.

In addition, when these viruses can be transmitted or occur naturally in different species of animals, they produce similar neoplasms within each group.

In other words, in the case of these generic groups of viruses, we have a situation in which one of the viruses, or many, in a group may cause cancer, but not necessarily all of them.

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[1] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

The papovaviruses are shown in table 1. We have polyoma virus, Shope papilloma, bovine papilloma, human papilloma, canine papilloma, oral papilloma virus, and the SV40 vacuolating virus. All are transmissible. Polyoma, SV40, and bovine papilloma grow in various species; the rest appear to be relatively host-specific.

TABLE 1.—Animal tumor viruses

Virus	Natural host	Other hosts	Nucleic acid type	Viral inclusions
"Papova" group:				
Polyoma...........	House mouse....	Hamsters, rats, rabbits.	DNA	Nucleus
Shope papilloma.....	Cottontail rabbits.	Domestic rabbits.	"	"
Bovine papilloma....	Cattle...........	Horses, hamsters.	"	"
Human papilloma...	Man............	?	"	"
Canine oral papilloma.	Dogs............	?	"	"
SV40 (vacuolating virus).	Monkeys (rhesus).	Man, hamsters, mastomys.	"	"

Fortunately many of these viruses are not malignant, and until now they have not been of much concern in relation to cell culture and vaccine.

The adenovirus group is shown in table 2. Here we have the human adenoviruses. Three are highly oncogenic. Five are intermediately oncogenic.

We have recently added a group C which contains types 1, 2, 5, and 6, which are oncogenic in the sense that they transform rat cells in tissue cultures. This is being reported by Dr. A. Freeman and several other associates.

There are now some 10 simian viruses that have been isolated from rhesus and African green monkey kidneys, and African green monkeys themselves, which cause tumors in hamsters.

In the poxvirus group are rabbit fibroma, rabbit myxoma, squirrel fibroma, and monkey facial tumor virus occurring in many different species. One could add to this viruses that Shope demonstrated in deer. As one can see, there is not just one virus that causes cancer, but many representatives.

Many RNA viruses cause cancer in their natural hosts. What I want to bring out here is the fact that the avian and murine sarcoma viruses have many similarities (table 3). They actually must belong to the same generic group if not the same species group of viruses.

Avian and murine sarcoma viruses both produce focus formation. They each have a defective sarcoma genome easily demonstrated which is easily rescued with the leukemia virus both in tissue culture and in the animal itself.

They have type-specific envelope antigens and a group-specific antigen.

They replicate by budding at plasma membranes.

TABLE 2.—Animal tumor viruses

Virus	Natural host	Other hosts	Nucleic acid type	Viral inclusions	Comments
Adenovirus group:					
Human:					
Group A—Types 12, 18, 31	Man	Hamsters and rats develop cancers.	DNA	Nucleus	Other adenoviruses apparently not oncogenic.
Group B—Types 3, 7, 14, 16, 21	"	"	"	"	Oncogenic behavior is correlated with tumor antigen DNA structure and mRNA which are specific for each of the two groups.
Simian:					
6–7/18 types oncogenic	Monkey	Hamsters	"	"	Virus-specific tumor or "T" antigens have been demonstrated for all but the avian adenoviruses.
Bovine:					
1/3 types oncogenic	Bovine	"	"	"	
Canine:					
Canine hepatitis	Dog	"	"	"	
Avian:					
1/2 strains oncogenic	Chicken	"	"	"	
Poxvirus group:					
Rabbit fibroma	Rabbit	Squirrel	"	Cytoplasmic	Studies by Duran-Reynals suggest that vaccinia can potentiate tumors caused by chemicals.
Rabbit myxoma	"	?	"	"	
Squirrel fibroma	Squirrel	Rabbit, woodchuck	"	"	
Monkey facial tumor	Monkey	Man?	"	"	

TABLE 3.—Similarities between avian and murine sarcoma viruses

	Avian	Murine
Focus formation	+	+
Defective sarcoma genome	+	+
Rescue with leukemia virus:		
In vitro	+	+
In vivo	+	+
Antigens:		
Type-specific envelope	+	+
Group-specific (gs) internal	+	+
Replication by budding at plasma membrane	+	+
Host range (determined by helper virus envelope)	+	+
Pathogenesis determined by sarcoma genome not by helper virus	+	+
Interference by leukemia viruses with sarcoma virus	+	+
Natural occurrence	+	+
Development during passage of leukemia viruses	+	+
Transfer of genome from nonproducer sarcoma cells to normal cells without helper virus	+	−

They have a host range determined by the helper virus envelope.

They have a pathogenesis determined by the sarcoma genome but not by the helper virus.

Interference by leukemia viruses with sarcoma virus occurs in both cases.

They occur naturally and produce cancer naturally.

Sarcoma virus also has emerged during the passage of leukemia viruses. In other words, pathogenic mutants of leukemia viruses can cause sarcoma, and you have transfer of the genome from nonproducer sarcoma cells to normal cells without helper virus. This occurs in the avian system. It has not occurred so far in the murine system, despite the use of radiated Sendai and other viruses to cause heterokaryons. So far we haven't been able to transmit murine sarcoma virus genome without the helper virus.

There are other viruses that have not been established as cancer viruses but are very often associated with cancer both in man and in animals. These include the herpesviruses and related cytomegalovirus and varicella.

Perhaps the most interesting one at the moment is the Burkitt herpes-type particle which is found, as I am sure Dr. Henle will report later, in human lymphoma cells, both in Africa and in this country.

It has been established that this herpes-type particle, which is defective in that it can't be transmitted readily by cell-free extracts or by filtrates, is not related to herpesvirus hominus or to other human herpes-type viruses. It does not appear to be related to cytomegalovirus or varicella, although I am not sure that enough work has been done to rule this out.

The question of whether or not the defective Burkitt particle or any of the other nondefective herpesviruses, including cytomegalovirus and varicella, can cause cancer has not been determined. There is no evidence that any of them has caused transformation or has produced oncogenic effects in experimental animals despite many opportunities both in the laboratory and in nature.

The question of neoplastic DNA viruses in relation to human cancer is one that really hasn't been resolved, because except for papilloma in certain circumstances and certain pox viruses, these viruses do not cause much cancer, if any, in their natural hosts, at least in the way that we have been observing them.

We cannot, however, be sure that these viruses which can be detected and eliminated from vaccines do not have a defective or latent state which might still somehow be involved.

In any case, these viruses can be observed and detected and eliminated from tissue culture systems and for this reason are possibly of less concern from the vaccine point of view than viruses that are covert, such as the RNA viruses which I will discuss later.

Table 4 illustrates adenovirus oncogenesis. The group which is highly oncogenic, and the one which is poorly oncogenic in hamsters, have different T antigens as well as different DNA composition and G + C content.

As I mentioned before, we have a C group that includes types 1, 2, 5, and 6, which transform rat cells and which contain a third group of T antigens, a group-specific messenger RNA, and a different G plus C content as well. Dr. Green will discuss this later.

The other adenoviruses have been checked now in hamster and rat cells, and so far they have not been found to be oncogenic in these systems.

Types 1, 2, 3, and 5 are now known to be oncogenic in rat cells, and like other serotypes leave behind a virus-free transformed cell containing T antigen and specific messenger RNA. These viruses are very common in children.

Text-figure 1 shows the prevalence of adenovirus type 2 antibodies in children as early as 2 years of age. Nearly 80% have antibodies to type 2.

Type 3 must now also be up here in the 70 or 80% category because it has been very prevalent in recent years.

Table 5 shows specificity of adenovirus group C. Types 1, 2, 5, and 6 react only with their own group and not with 4, nor with the A or B group.

The simian adenoviruses—which may have some interest since they haven't been looked at at all in relation to human cancer—occur in monkey kidneys which have been used rather extensively for production of human vaccines and a number of simian adenoviruses are oncogenic as well. Types 1, 11, 25, 33, and 38 have a tumor antigen that appears to be specific (table 6). This grouping was suggested by Dr. R. Gilden.

Group II. This contains SV20, possibly SV23.

Group III contains SA–7. So we have in the oncogenic simian adenoviruses apparently three different groups based on the tumor antigen produced by the tumors induced in hamsters.

I mentioned that the DNA viruses are not very impressive from the standpoint of our knowledge about their ability to cause cancer in their

Table 4.—Adenovirus oncogenesis in relation to DNA and mRNA antigenic determinants

T antigen group*	Type	Oncogenicity†	HA group‡	DNA composition G + C content§	mRNA¶
A	12	High (A)	4	Low (47–49%)	A
A	18	"	4	"	A
A	31	"	4	"	A
B	3	Moderate (B)	1	Intermediate (50–53%)	B
B	7	"	1	"	B
B	14	"	1	"	B
B	16	"	1	"	B
B	21	"	1	"	B
B	11	Negative (C)	1	"	Unclassified‖
B	4	"	3	High (56–60%)	"
Unclassified**	1, 2, 5, 6	"	3	"	"
"	8–10, 13, 15, 17, 19, 22–24, 26, 27, 29, 30	"	2	"	"
"	20, 25, 28	"	12	"	"

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*See table 2.

†Based on tests in newborn hamsters observed for 400 or more days, except for types 8, 9, 10, and 27 which have been on test between 250 and 300 days.

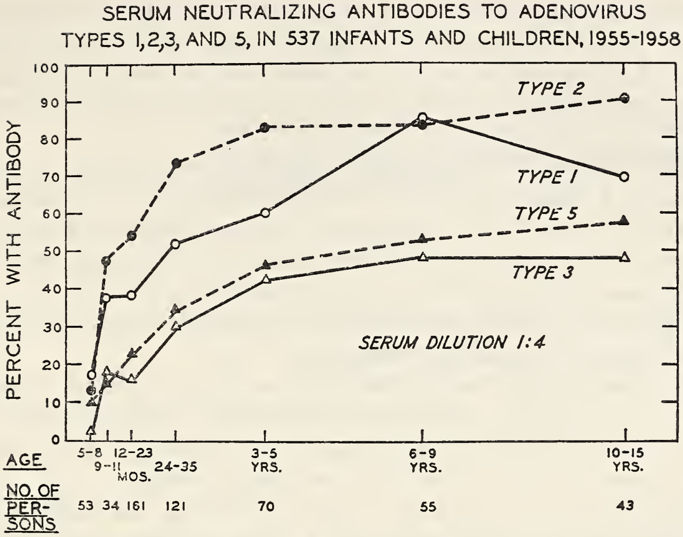
‡Hemagglutinin groupings.

§ Guanine + cytosine content.

¶Messenger RNA isolated from ribosomal fraction of hamster tumors induced by the respective viruses grouped according to hybridization with purified DNA of various adenoviruses.

‖Messenger RNA's from tumors induced by type 12 (RNA group A) and by type 7 (group B) were found nonreactive with DNA's of types 2 and 4.

**Not in T antigen group A or B.



TEXT-FIGURE 1.—Serum-neutralizing antibodies to adenovirus types 1, 2, 3, and 5 in 537 infants and children, 1955–58.

TABLE 5.—Specificity of adenovirus group C — T antigens and sera

Virus type*	1—SV40 or 2—SV40	SV40	AD7	AD12
1, 2, 5, 6	≥64†	<2	<2	<2
4	<2	<2	8	<2
3, 7, 11, 14	<2	<2	16–32	<2
12	<2	<2	<2	16
9, 10	<2	<2	<2	<2
KB cell control	<2	<2	<2	<2

*20% cell suspensions of KB cells harvested at maximum cytopathic effect. Group (Hexon) antigen titers of 128.
†CF titer reciprocals with 4–8 units of the above tumor antiserum.

TABLE 6.—Serological grouping of oncogenic simian adenoviruses

Designation	Virus types*	Based on	
		Tumor antigens	T antigens
I	SV1, 11, 25, 33, 38	+	+
	34	Not tested	+
II	SV20 - (SV23)	+	Negative
III	SA-7	+	Negative

*SV37 remains ungrouped.

VIRUS VACCINE PRODUCTION

natural hosts. Tumors which are produced are benign, and the others apparently remain unexpressed in nature within the limited life of feral animals. And one of the ways I suppose that we are all trying to solve cancer is to postpone it until we die of something else before we die of cancer.

This is what happens in mice. It happens in other species as well.

However, I believe the RNA viruses are more of a problem for two reasons: First, the RNA myxoviruses in the chicken and the mouse do cause cancers and leukemia in their natural hosts. They also are very often covert in these animals and in tissue cultures derived from them.

It appears my time is up and I will not be able to document the RNA viruses. You will read about them later.

What I was going to say is that it appears that the RNA viruses in the mouse and the chicken are the most important causes of cancer in these animals when they are kept to old age; and they are, as far as we can tell, the most prevalent viruses for both species of animals.

This isn't surprising because, with the RIF test, the COFAL test, and the COMUL test, my associates and I have been able to isolate literally hundreds of leukemia viruses from normal animals of these species. In doing this we have found that in almost every case the question of their presence or absence could not have been determined in the normal animal without the new techniques recently developed for this purpose.

I won't have time to talk about these. I can only say that the methods have been published recently describing the COMUL test for the murine viruses and, of course, the COFAL test for the avian virus had been reported several years ago. I refer you to these publications.

Viral Oncogenesis and Results of Inoculation of Normal and Transformed Cells Into Nonhuman Primates[1]

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ONE problem central to the subject of this working conference is that of choosing the most *suitable* cell culture system for the production of live virus vaccines. I will discuss two of the many questions which should be posed in making this choice. Are those animal viruses that can induce tumors any real danger for man if he is immunized with vaccines containing such viruses or is this only an academic hazard? And, what is known of the potential *in vivo* tumorigenicity of altered or unaltered cells grown in culture?

Contrary to original belief, it is now well established that "oncogenic" or tumor-inducing viruses are not highly species specific. Viruses cytopathic for cells of one species may transform "normal" cells of another species to "altered" or even to true "neoplastic" cells, and latent viruses carried *in vivo*, when inoculated into individuals of another species, may produce true neoplasms. One group of such agents is the avian leukosis viruses, one of which, Rous sarcoma virus (RSV), in certain strains will induce tumors not only in the original avian hosts but also in species as far removed as reptiles and mammals including the primates (*1–8*).

In our studies we induced metastasizing immature sarcomas in one species of marmosets (small South American nonhuman primates) by inoculation of $10^{4.5}$ or more focus-forming units (FFU) of the Schmidt-Ruppin strain of RSV (SR–RSV) and in a preliminary experiment in 1 of 4 ani-

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[1] This investigation was supported in the main by Public Health Service research contract PH 43–62–179 from the National Cancer Institute, Special Virus Leukemia Program, and partly by Public Health Service training grant I–TI–AI 253–03 from the National Institute of Allergy and Infectious Diseases and by research and education funds, Presbyterian-St. Luke's Hospital, Chicago, Ill.

mals by inoculation of the Bryan high-titered strain of RSV containing avian myeloblastosis virus as a helper (BR–RSV–AMV).[2]

All marmosets inoculated with SR–RSV at birth or up to 6 months of age, regardless of the age of the animal at the time of inoculation, developed metastasizing sarcomas after a latent period of 3 weeks to 2 months, without any definite correlation between the age of the animal at the time of inoculation and the length of the latent period. The effects of inoculating animals older than 6 months are being studied, but it is too early for results. In contrast, the 1 sarcoma induced by BR–RSV–AMV developed after a much *longer* latent period of 12 months. The tumors induced by the SR–RSV could be transplanted between identical or chimeric twins but not to unrelated animals, either newborn or adults.

Infectious SR–RSV was recovered from all SR–RSV marmoset tumors that have so far been examined. These tumors did not yield virus if tested by direct inoculation of live cells or cell extracts obtained either from the tumor at biopsy or autopsy or after up to 20 cell culture passages. But virus was regularly recovered if tumor cells were "co-cultivated" with chick embryo cells free of leukosis viruses, as determined by the absence of COFAL antigen and lack of interference with other leukosis viruses (*9*). Neither test, unfortunately, will detect the presence of either the agent of Marek's disease or maybe any other as yet unknown members of this group of viruses. More recently, Dr. Barbara Marczynska of our group has been able to induce SR–RSV marmoset tumor cells grown in cell culture to produce infectious RSV by the addition of filtered extracts of "leukosis-free" chick embryo cells. Dr. Marczynska's unexpected results are now under extensive study in our laboratories.

Electron microscopic studies of the SR–RSV marmoset tumor cells by Dr. Roger Smith of our group have demonstrated a unique complex of repeating cytoplasmic membranes which contain particles resembling avian leukosis viruses at different stages of completion, but no budding particles were demonstrated (*10*). These structures were found in all tumors induced by SR–RSV, but not in the sarcoma induced by BR–RSV–AMV, nor in a liposarcoma which developed in an animal treated with a chemical carcinogen and vaccinia virus a year before the appearance of this tumor, nor in a variety of normal marmoset tissues.

COFAL antibodies were detected only in the 1 animal developing a sarcoma 1 year after inoculation with BR–RSV–AMV. COFAL antigen was not detectable in any of the tumors induced by SR–RSV; the 1 tumor induced by BR–RSV–AMV has not yet been evaluated.

Chromosomal analysis of cells from the original marmoset tumors or tumor cells after up to 20 cell culture passages has shown normal marmoset karyotypes or various randomly distributed chromosomal aberrations without the predominance of a specific change which could be taken as a marker.

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[2] The experiment with BR–RSV-AMV was performed in collaboration with Dr. B. R. Burmester, Regional Poultry Research Laboratory, U.S. Department of Agriculture, East Lansing, Mich.

This documents the results from our laboratory which have any relevance to my first question but I will leave the discussion of these findings until the end of this presentation and move to the second question—that of the potential tumorigenicity of altered or unaltered cells grown for many generations in cell culture. Since time is insufficient for discussion of the entire subject of "spontaneous" and/or "induced alterations" of normal cells in culture, I will only present some of our own results for discussion.

Cell cultures were started from a marmoset kidney obtained by unilateral nephrectomy; the kidneys were grown in cell culture by standard techniques. Some of these cultures were exposed to SR–RSV but no changes in cell morphology, cellular growth rates, or chromosomal complement were observed. Inoculated and control cultures were subcultured every 3–5 days for more than 160 cell passages. The cells were observed regularly for changes in their morphology and their growth patterns and were karyotyped approximately every 5–10 cell passages. The original cultures were split into several lines which were kept separately but handled identically. Some of these cell lines stopped growing after 50–100 cell passages, and this was generally associated with extensive chromosomal aberrations in the cells of the dying line. However, 3 of these lines have continued to grow and are now beyond 160 cell culture passages. Of these, 2 are morphologically altered and exhibit extensive chromosomal aberrations both in chromosome structure and chromosomal numbers but have maintained contact inhibition. The cells of the third line are also morphologically altered and have lost contact inhibition, but about 70–80% of the cell population have a normal karyogram (table 1). All these lines were inoculated into the autologous host; *i.e.*, the same animal from which the kidney had been removed for the initiation of these cells lines. No tumors were observed at any of the inoculation sites, and cells from all 3 lines failed to grow in hamster cheek pouches (*11*).

TABLE.—Characteristics of three marmoset cell lines at the 160th passage level

	Morphology	Contact inhibition	Karyotype
A	Altered	Yes	Abnormal
B	Altered	Yes	Abnormal
C	Altered	No	Normal diploid (70–80%)

I will now consider the two questions I posed at the outset. The data on RSV-induced metastasizing sarcomas in nonhuman primates must be taken as evidence for the real danger of at least one animal tumor virus for man. Nonhuman primates inoculated as late as 6 months of age with as little as $10^{4.5}$ infectious doses of RSV regularly developed true neoplasms, and if we can succesfully transfer this virus from its natural avian host across the species barriers to nonhuman primates, there is little doubt in my mind that man would be equally susceptible.

Investigations relative to our second question are numerous. It has been pointed out many times that the criteria for alteration of cell characteristics *in vitro* do not provide definite markers for the tumorigenicity of such cells *in vivo.* In our own experiments none of 3 morphologically altered cell lines, with and without loss of contact inhibition and with and without chromosomal changes, produced tumors in the autologous or heterologous hosts. But RSV-induced tumor cells, which likewise had an altered appearance *in vitro*, were truly neoplastic when inoculated into naturally tolerant homologous animals. Until clear evidence to the contrary is available, I believe that we must consider all viruses known to cause neoplastic growth in any species as real potential hazards for man and any marker of abnormality in cultured cells as a possible indicator of *in vivo* tumorigenicity. But the weight to be given to either of these beliefs, particularly to the latter, must obviously be balanced against the value of the vaccine.

Finally, I would like to make one suggestion for discussion. Whenever primary cells are used to prepare a live virus vaccine, we use cells from a number of animals. With each cell batch we encounter the chance of contamination with latent, potentially oncogenic agents. On the other hand, if we use diploid cell lines of human origin, we could exclude contaminating agents to a much greater extent. A particular candidate cell line could be frozen and tested by every available technique for contamination with oncogenic or any agent for any length of time, an extent of testing which would be impracticable for testing each new batch of primary cells. But one doubt would still remain—perhaps even unaltered diploid human cells, intact or extracted, could become tumorigenic if inoculated into man.

Since this remote possibility cannot be tested by inoculating man, the use of diploid cell lines of animal origin could provide an additional safeguard. If tissues were surgically removed from an animal, then the cell lines to be used ultimately for vaccine production could undergo the same extensive testing procedures suggested for human cell lines and tests for tumorigenicity not only in heterologous species but also in homologous and autologous animals. If human cell lines are ultimately selected as substrate for the production of live virus vaccines, more studies similar to the one conducted in our laboratories on marmoset cell lines would at least increase our confidence in the safety of diploid cell lines derived from man.

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VIRUS VACCINE PRODUCTION

Viral Oncogenesis and Results of Inoculation of Normal and Transformed Cells Into Nonhuman Primates—Discussion

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DR. Deinhardt's studies of various Rous sarcoma virus strains in nonhuman primates are important because they demonstrate the ability of tumor viruses to cross species' barriers and infect species phylogenetically far removed from each other. Since this finding appears to be consistently reproducible, this model system should provide much information of value over the next few years.

For the purposes of this Conference, however, the failure, to date, to induce leukemia or lymphoma in hundreds of newborn mother-deprived primates, inoculated on the day of birth with prime clinical material from acute human leukemia, poses the question of the real versus theoretical risk in the use of human-derived cells.

Recent work by Huebner (*1*) and co-workers on the Moloney sarcoma-murine leukemia complex, which shows a striking comparability to the RSV-avian leukosis system, gives rise to the hope that a similar relationship will be uncovered in a human system and eventuate in means to detect and control such agents. These studies deserve the highest priority and are being investigated vigorously.

But even if one had such tests available now, the specter of yet undiscovered tumor viruses will always hang over the development of vaccines for human use. Dr. Deinhardt's suggestion, that diploid strains from nonhuman primates be used for vaccines, has merit in that exhaustive testing could be performed with such cells, including inoculation of cells back to the donor.

Comparative virology has shown so far that every animal species studied sufficiently has at least one oncogenic adenovirus, and one also gets the impression that further study will demonstrate a variety of sarcoma-leukemia complexes in a number of species.

It is inconceivable to me that a system will be found which is completely free of any potential risk; so the real problem is one of minimizing the risk by applying all of the monitoring and assay techniques which we now have or may develop in the future.

It will also place a heavy burden of responsibility on the control authority which has to approve the use of such vaccines, and as Dr. Enders has so aptly said ". . . to determine if the game is worth the candle."

Development of fractionated, nucleic acid-free, viral antigens would appear to be the bright hope of the future and the needed technology is now being developed.

Perhaps 5 years from now we will look back and remember this period as the end of the "horse-and-buggy" days of vaccines.

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GENERAL DISCUSSION

Dr. P. K. Vogt: Dr. Deinhardt, how do you protect the people working in your laboratory from RSV?

Dr. F. Deinhardt: We protect them the same way we do against poliomyelitis or any other infectious agent.

Dr. J. P. Fox: Dr. Deinhardt, what was the serologic response, if any, of the marmosets infected with the RSV virus strains?

Dr. K. Habel: Which kind of serological response? Antitumor or antiviral? Or both?

Dr. Fox: Antiviral on one hand, or antitumor.

Dr. Deinhardt: In our initial experiments, in which animals were inoculated at birth, we barely got any sera, because marmosets at birth weigh only 32–42 g, and the animals were still very little when they died from their malignant disease 35–60 days later. In more recent experiments we inoculated older animals, which were bled regularly, but these sera have not been fully evaluated. However, the one animal which developed a tumor about 1 year after inoculation with the Bryan RSV strain, which we obtained from Dr. Burmester, had a COFAL antibody titer of 1:128.

None of the sera have been evaluated yet for neutralizing antibody to RSV, and it is too early to make any definite statement about the results of fluorescent antibody studies which have just begun.

Dr. Habel: Perhaps we should say that the COFAL test as I understand it now, Dr. Huebner, is measuring the GS antigen?

Dr. R. J. Huebner: Yes.

Dr. Habel: This is the internal antigen of the virus particle.

Dr. F. Rapp: Dr. Stevenson is one of a number of people who has mentioned the possibility of purifying the viral protein free of nucleic acid for vaccine production. This raises, of course, the old issue of live versus inactivated vaccines and raises the specter of multiple inoculations of such inactivated protein. I expect there are adherents to both principles here.

It is more than just purifying the protein. If one wanted to make a very long-range forecast, one might say one could isolate that piece of the viral genome responsible for late antigen synthesis which is presumably, so far as we know, not responsible for the oncogenic properties of the virus, and inoculate that.

I don't recommend this. But if one is forecasting from a long-range point of view, this makes quite as much sense perhaps as thinking in terms of entirely purified protein.

Dr. Habel: Provided that particles with such a defective genome were infectious.

Dr. P. Long (U.S. Department of Agriculture, Regional Poultry Research Laboratory, East Lansing, Mich.) : Dr. Deinhardt, which Schmidt-Ruppin did you use in your marmosets? Number 1 or 2?

Dr. Deinhardt: We used the standard SR–RSV preparation, which was produced for the National Cancer Institute by Dr. E. H. Bernstein of University Laboratories, Inc., Highland Park, New Jersey. This virus stock has not been analyzed in genetically controlled chick tissues and it is not known if it is "1," "2," (of "A," "B") or both.

Dr. Habel: Does that answer your question?

Dr. Long: No.

Dr. Habel: It doesn't? Then state it again in a different way perhaps.

Dr. Long: Schmidt-Ruppin 1 is A type. Schmidt-Ruppin 2 is B, subgroup 5. I wonder which one Dr. Deinhardt found to be infectious in marmosets.

Dr. Habel: You don't know whether this was A or B? Which type of coat was it? Avian myeloblastosis virus 1 was the helper responsible for the high-titer Bryan envelope.

Dr. Huebner: It was probably B.

Latent Viral Infections in Donor Tissues and in Recipients of Vaccines[1]

JOSEPH L. MELNICK, *Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas 77025*

EVEN though I have been invited by the Organizing Committee to talk about latent viruses, I presume they really intend for me to discuss latent infections caused by viruses. Consequently, some definitions are in order, and it may be pertinent to restate the definitions agreed to at the Madison Conference (*1*) held 10 years ago.

> An inapparent infection is a primary infection evolving without symptoms and usually leaving a lasting immunity. Regardless of whether the primary infection evolves into a disease or remains inapparent, the virus in some cases may persist. If the virus persists in the absence of symptoms, the infection is *latent*. In a latent infection, the virus might or might not be in a proviral or in a vegetative state, and virus particles might or might not be produced.

It is well known that bacterial viruses achieve latency by becoming attached to, or integrated with, the genome of their host cells. Such a provirus can remain undetected in a bacterial cell line for countless generations, except for a rare accident that upsets the balance and leads to virus production. When this happens, virus parts are made and assembled, and the cell dies as infectious virus is released.

In view of the current interest in viral oncology, it is important to remember that bacterial viruses can induce new antigens in the cell, particularly at the surface. When the virus is eliminated from the cell, the new antigens disappear also.

Sometimes the provirus genome is defective. Under such circumstances, it cannot be recognized unless a helper virus is added to correct or compensate for the defect in the provirus genome. Because the defective virus can induce synthesis of new cell surface antigens by means of lysogenic conversion, a resultant defective virus can be responsible for certain

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expressions of the cell phenotype. However, without the helper virus, we would never know whether the genetic information for producing certain cell surface antigens resides in host or viral DNA. Recent experiments (*2–4*) with cell fusion in which a virus-free cancer cell may be induced to yield infectious virus offer new leads in which helper viruses plus susceptible cells may be fused with cancer (and other) test cells to determine if the test cells contain defective viral genomes.

LATENCY IN DONOR TISSUES

Earlier speakers at this Symposium have discussed the viral flora of tissues used as the source for cell cultures. I have been asked to consider latency in such donor tissues.

Avian Tissues

Most flocks of chickens have been found to be infected with the various leukosis viruses, especially visceral lymphomatosis virus. The virus is usually introduced early in life when respiratory vaccines contaminated with leukosis virus are inoculated into baby chicks. The virus is transmitted horizontally through the saliva and feces, producing an infection in the adult chicken characterized by transitory viremia and lasting antibodies. Relatively few adult birds develop obvious disease. Vertical transmission has been demonstrated from the viremic hen, but not from the viremic rooster. This results in congenitally infected viremic chickens, tolerant to the virus, free of antibodies, and permanent shedders of the virus (*5*). The incidence of leukemia in congenitally infected animals is much higher than in animals infected by contact. Apparently this virus which is so widespread, and which acts as a helper for Rous sarcoma virus, has actually replaced the Rous virus in present-day chicken flocks. Since avian leukosis virus can also interfere with Rous sarcoma virus, it seems to have effectively "vaccinated" chickens against Rous sarcoma. However, the cure may be worse than the original disease.

When cells are cultured from chick embryos obtained from infected flocks, they appear normal, even though they contain the leukosis virus. Virus replication can be detected in a number of ways: 1) interference with the focus-forming capacity of Rous sarcoma virus, 2) serological tests for the protein coat of the leukosis virus, and 3) serological tests for the group antigen with the use of hamster or other mammalian sera from animals carrying tumors induced by the Schmidt-Ruppin strain of Rous sarcoma virus. Such tumors do not possess infectious virus, and thus the animals do not develop antibodies against the virus coat protein.

The avian leukosis viruses induce such widespread latent infections that chick embryos must be obtained from virus-free flocks if they are to serve as donor tissues for vaccines used in man. This is true for measles vaccine.

However, for some years yellow fever vaccine has been made in leukosis virus-infected cultures, but perhaps this is no longer allowable. Influenza vaccine is still prepared in leukosis virus-infected cultures, but both viruses are inactivated in the process.

As of this date, Miller's epidemiological studies from the National Cancer Institute have not revealed any unusual increase in leukemia or other malignancy in the large number of troops who received yellow fever vaccine during World War II.

Canine Tissues

Dog cells may also be used for preparing vaccines for human use. Canine adenovirus that causes hepatitis has been repeatedly isolated from canine kidney cultures. Because of frequent occurrence of canine lymphosarcoma, caution should be exercised, particularly in view of Huebner's recent findings with murine sarcoma (*6*). The situation for the murine sarcoma is precisely that for the avian sarcoma. Even though no virus can be detected by ordinary means, the addition of the proper helper virus supplies the coat protein to the sarcoma virus genome and allows it to be detected. What is needed to determine whether these findings apply to the dog is the correct helper virus. If the situation parallels the avian and murine species, noncytopathic leukosis viruses may be common in many cells of species in which sarcomas naturally occur. One way to detect such viruses in the tissue of the host would be to look for a group-specific antigen in the cells of the donor tissue, with the use of the serum of the animal carrying the sarcoma. Thus one might be able to detect directly the group-specific antigen for a canine leukosis virus.

Simian Tissues

Monkey kidney tissue has been used most widely in the preparation of vaccines for human use. A considerable number of viruses have been recovered from monkeys, but we shall be concerned here only with those producing latent infection in kidney cell cultures.

A number of investigators have demonstrated a balance between viruses and cells maintained in tissue culture. Such cultures sometimes show cytopathic effects, but surviving cells may grow out and continue to shed virus continuously or intermittently for long periods. The discussion of the role of interferon in establishing this equilibrium has been assigned to others at this Conference.

Some viruses have appeared repeatedly in monkey kidney cultures. Some, like herpes B virus, paramyxovirus SV5, foamy virus, and papovavirus SV40, can be detected by seeding culture fluids on susceptible cells. Then detection is straightforward—by the appearance of a specific cytopathic effect leading to specific virus identification with specific antiserum. A number of these potentially contaminating viruses from monkey kidney cells may be suppressed if the cells are grown in a medium containing a high concentration of aluminum chloride (*7*).

Another problem has recently arisen—the incorporation of a defective SV40 viral genome present in monkey kidney cell cultures into the seed virus used for preparing adenovirus vaccine (*8–10*). During this interaction, the vaccine virus may become capable of replication to much higher titers in the monkey kidney cells, but in acquiring this, it may also have enhanced its oncogenic potential. However, this is not always the case. Recent studies (*11*) have shown that transcapsidation of defective SV40 genome into some adenoviruses may make them *less* oncogenic (*see* table 1).

TABLE 1.—Adenovirus subgrouping by nucleic acid and oncogenicity

Virus subgroup	DNA % G-C ratio *	Hamster oncogenicity †	Tumor mRNA hybridization ‡		SV40-adeno hybrid oncogenicity §
			From subgroup A tumors	From subgroup B tumors	
A	Low (47–49%) (types 12, 18, 31)	+++	+	0	
B	Intermediate (50–53%) (types 3, 7, 11, 14, 16, 21)	+	0	+	Virtually none (except type 7)
C	High (56–58%) (types 1, 2, 4, 5, 6)	0	0	0	High (types 1, 2, 5, 6)

* From Green *et al.*

† From Huebner, Trentin, Hilleman *et al.*

‡ From Green *et al.* Hybridization with DNA of viruses belonging to subgroup A, B, or C as indicated.

§ From Rapp, Melnick *et al.* PARA-hybrids indicated were prepared by transcapsidation from parent SV40-adeno 7 hybrid population.

The defective genome in the adenovirion may be a piece of SV40 genetic material—sometimes enough to code for the entire infectious SV40 virion (*12*, *13*) or sometimes only enough to code for T (*9*, *10*) and transplantation antigens (*14*). Other examples of the acquisition of new genetic material are known in which the adenovirus acquired increased infectiousness for formerly resistant cells (*15*). Adenovirus containing contaminated genetic materials has been recognized by an analysis of infection kinetics required for plaque formation or by the induction of synthesis of new antigens not specified by the adenovirus genome (*15–17*).

Another defective virus has recently appeared in monkey kidney cultures—adeno-associated satellite virus. It may be present and passed from cell to cell, but for its development into a recognizable virion, adenovirus must be added to the culture (*18–20*). Present evidence suggests that all four known types of satellite virus occur in monkeys (*18*, *21*, *22*) but that only types 1–3 occur in man (*22*).

Because of the number of viruses that have been isolated from monkeys, it is worthwhile calling attention to a study carried out 10 years ago on cytologic aberrations in primary cultures of normal monkey kidney cells (*23*). The types of aberrations are illustrated in the figures. Figure 1 shows two normal metaphase figures. In figures 2–13 are mitotic aberrations, such as multipolar spindles, anomalous spindles with scattered chromosomes, lagging or precocious movement of chromosomes, chromosome fragments, and chromosome bridges; these were found in all cultures with a frequency ranging from 16–40% of the dividing cells. Figures 14–23 show nonmitotic aberrations, such as giant nuclei, multinucleate cells, nuclear fragmentation, micronuclei, nuclear inclusions, and phagocytosis; these were found in approximately 3–7% of the nondividing cell population. Despite the high frequency of these aberrations, we know little as to whether they reflect latent viral infections. Virus vaccines prepared in such cultures have been administered to many millions of person, by mouth, as live virus preparations or, by injection, as inactivated virus vaccines, and no evidence is available of any untoward effects.

LATENCY IN MAN

This part of my discussion can be brief—not because latent infections do not occur in man, but because viral vaccines are not known to influence this condition when it exists, and certainly there is no direct evidence that vaccines have initiated a latent infection. This seems to be true, even though contaminating viruses have inadvertently been given to many persons. Thus live avian leukosis virus has been injected along with yellow fever vaccine; live simian papovavirus has been administered orally along with Sabin poliovaccine and has been injected together with Salk poliovaccine and also with adenovirus vaccines. Gerber (*22*) has shown that children receiving contaminated Salk vaccine may develop SV40 antibody levels that remain constant for at least 3 years in the face of a progressive decline in polio antibodies. This difference in response to the two viral antigens suggests the presence of a continual SV40 antigenic stimulus. Previously, infants receiving contaminated Sabin vaccine had been found to excrete SV40 for as long as 5 weeks (*25*).

For completeness, I shall mention the known latent viral infections of man. The earliest to be recognized is that of herpes simplex. Primary herpes simplex infection is short lived—the virus and the lesions disappear only to reappear at frequent intervals, despite the presence of serum antibodies. The condition of herpesvirus in the body during the latent infection is unknown.

Other members of the herpesvirus group also persist for long periods in the absence of disease. They may flourish in later life, stimulated by an unknown mechanism (zoster appearing years after varicella), or they may be activated by immunosuppressive treatments (zoster virus and cytomegalovirus). Latent adenovirus infections are common in man. This can easily be demonstrated by growing the adenoid tissue in culture and finding adenovirus in the cells that grow out. Recent work shows that a new herpes-type virus may also be detected in the same way in cultured Burkitt lymphoma cells. But this does not seem specific, for when peripheral blood cells are grown from healthy persons as well as from patients with various types of leukemia, the same herpes-type virus often appears in the cultured cells. As shown from the sero-epidemiological studies already carried out, infection with this agent, like infections with other members of the herpes group, is widespread in human beings (*26*).

Latency undoubtedly occurs in other virus infections of man. Two recently discovered examples are measles virus associated with subacute inclusion body encephalitis (*27*, *28*) and, particularly, congenital rubella. When rubella infection is acquired *in utero*, persistent infection may continue for up to 2 years after birth; virus also persists in cultures prepared from children who have been congenitally infected (*29*).

Other viruses causing latent infections in man are the satellites of adenovirus, whose growth depends on a late state in the replication of the helper adenovirus (*18–20*, *22*). Insufficient knowledge is available to discuss the effects of their potential activation by administration of live adenovirus vaccine.

RECOGNITION OF LATENT VIRUS INFECTIONS

I would like to call attention to methods in use for detecting viral latency in animal tissue culture systems:

1) Direct examination of cultured cells and tissue culture fluids in the electron microscope. Sensitivity can be increased at least 100-fold by examining preparations obtained by density gradient ultracentrifugation. The material banding between 1.16 (myxovirus) and 1.42 (satellite viruses) is concentrated and virions are more easily seen, since cellular debris is removed by the procedure. In this way, satellite viruses were first recognized in our laboratory.

2) Examination of cultures for infectious virus by passage to susceptible cells.

3) Examination of cultures for interfering properties against known viruses, particularly in avian tissue cultures, against the transforming action of Rous sarcoma virus.

4) Fusion of test cells with susceptible cells to determine whether this leads to expression of infectious virus.

5) Analysis of the infectivity dose-response curve to see whether it fits single- or double-hit kinetics. This would determine whether the virus seed has become contaminated with another genome from a known or unknown source in the tissue culture system in which it was grown.

6) Addition of helper viruses to the test cultures to determine if a new virus appears (satellite) or if a double-hit kinetic curve can be changed into a single-hit curve (PARA- and MAC-adenoviruses, murine sarcoma-leukemia viruses).

7) Tests of the donor tissue for the presence of group-specific antigens (such as those of avian leukosis virus) with selected sera that do not react with coat protein antigens.

8) Homology studies to determine if mRNA in the test cell hybridizes with nucleic acid of unknown viruses. This method has been applied for detecting mRNA of adenovirus and papovavirus in virus-induced tumors, and should also be applicable to cells carrying other incomplete viral genomes.

The speakers that follow will discuss the detailed applications of some of these methods to the problem of detecting viral latency and persistent infection of cells.

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"Normal" cultures of monkey kidney epithelial cells. All cells were stained with hematoxylin and eosin. × 700 [from (23)].

PLATE 25

FIGURE 1.—Two normal metaphase figures : A, polar view, B, side view.

FIGURE 2.—Tripolar anaphase figure.

FIGURE 3.—Tetrapolar metaphase figure.

FIGURE 4.—Tetrapolar metaphase with one group of chromosomes oriented in a plane perpendicular to another group. The metaphase plate seen from polar view contains only 12 chromosomes.

FIGURE 5.—Late anaphase of a tetrapolar division. *Note* unequal distribution of chromosomes to the 4 daughter cells.

FIGURE 6.—Unequal distribution of chromosomes and cytoplasm in a bipolar division.

FIGURE 7.—Hypohaploid cell in mitosis. Chromosomes appear thinner than typical metaphase chromosomes.

FIGURE 8.—Polyploid cell in metaphase. *Note* chromosome fragments which have failed to become oriented on the metaphase plate.

FIGURE 9.—Cell in mitosis showing precocious movement of chromosomes.

FIGURES 10 AND 11.—Cells in anaphase with chromosome bridges.

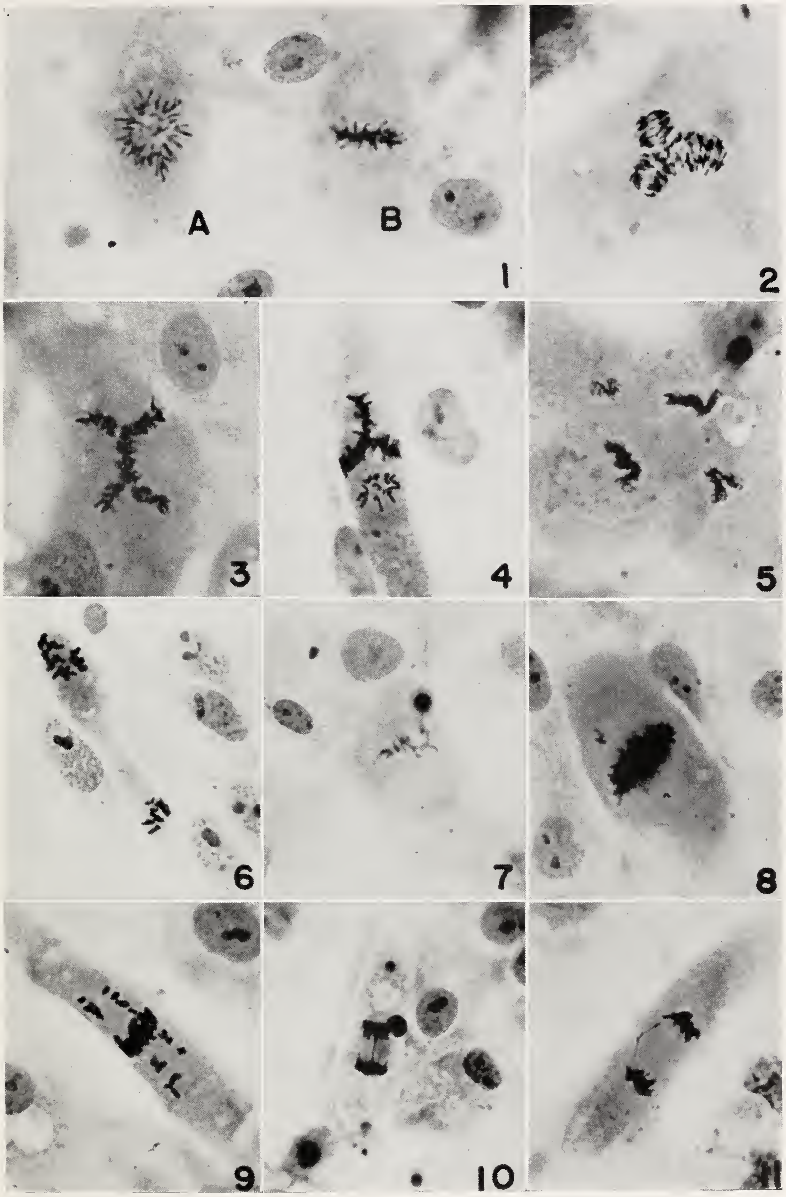
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PLATE 26

FIGURE 12.—Tripolar anaphase division figure with lagging chromosomes.

FIGURE 13.—Cell with anomalous spindle. Chromosomes are scattered about in a disorganized fashion.

FIGURE 14.—Multinucleate cell with 4 polyploid (or aneuploid) nuclei. The 2 nuclei on *right* have a common bridge between them and may be in the process of fusing. *Note* knoblike protrusion coming from nucleus at right. This is probably the manner in which most micronuclei, frequently seen in these cultures, are formed.

FIGURE 15.—Giant binucleate cell with extensive lobation of nuclei. Some interaction or connection between the 2 nuclei suggests fusion.

FIGURE 16.—Large nucleus containing a commonly seen inclusion, which appears to have been formed by an invagination of the nuclear membrane encircling cytoplasmic material.

FIGURE 17.—Cell showing a prominent nuclear inclusion bounded by dense basophilic membrane.

FIGURES 18 AND 19.—Nuclear inclusions which appear to form in association with invaginated surface of nuclear membrane. *Note* intricate system of radiations.

FIGURE 20.—Phagocytosis in a dividing cell. Phagocytized cell is pyknotic and dying, and has been completely engulfed by the dividing cell.

FIGURES 21–23.—Phagocytosis with nuclear involvement. Figures 21 and 22: Nucleus is encircling the phagocytized pyknotic cell. Figure 23: Nucleus has completely engulfed phagocytized cell ("nuclear phagocytosis"). Nucleoli (N) can readily be distinguished from phagocytized cell (I).

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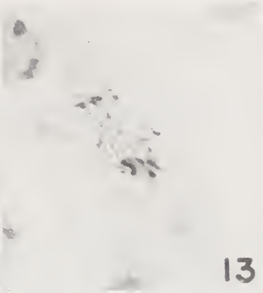

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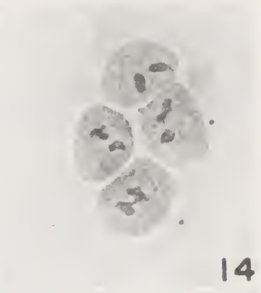

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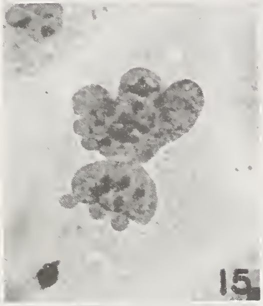

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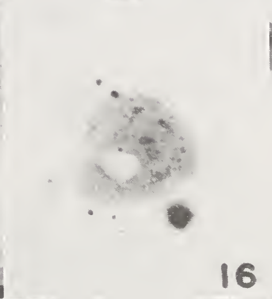

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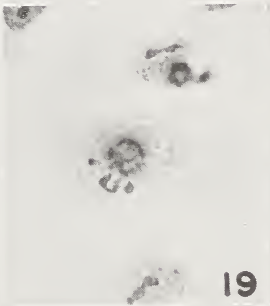

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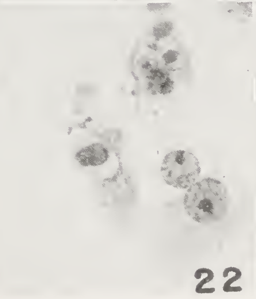

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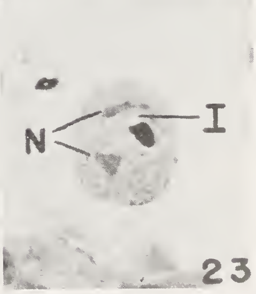

23

Detection of Latent Viruses in Kidney Tissues[1]

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The presence of latent viruses in cell cultures of both simian and nonsimian origin has been reported. The significance of these latent viruses in the donor tissues and the possible consequence resulting from the presence of these agents in the recipients of contaminated virus vaccines have been discussed by previous investigators (*1*, *2*).

During the past 4 years we have conducted a longitudinal survey study on viruses that occurred in monkey kidney tissues used for the preparation of primary cell cultures. To our surprise, an unusually high rate of virus recovery was made from cultures considered "normal" (*3*, *4*). One segment of this study is presented in table 1. From February 1966 through February 1967 a total of 417 lots of monkey kidney cells obtained from as many animals were cultivated and examined. These cultures were observed as long as the cells were in good condition. Of the 227 rhesus monkeys (RhM) obtained from India and 190 African green monkeys (GM), from Ethiopia, 225 showed virus infection in their kidney tissues. An average of 50% yielded one or more viruses each month regardless of the monkey species used or the season in which the tissues were obtained.

Although the prevalence of latent viruses in the so-called "normal" cell cultures was unusually high, these viruses were generally not recognized. The detection of latent viruses required special handling and long-term cultivation of the cell cultures. Examples are shown in table 2. Of the 86 lots of cultures examined, only 2–4% showed virus infection when the cultures were examined 14–21 days after the cells were planted—the usual period for most virological studies. However, when the same lots of cultures were examined 29–55 days after planting, a significantly higher percentage of cultures showed virus infection. In addition, the number of

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Table 1.—Prevalence of virus infections in monkey kidney cell cultures

Date	Rhesus monkeys			Green monkeys		
	Number studied	Virus infection		Number studied	Virus infection	
		No.	(%)		No.	(%)
1966						
February	13	9	68	9	6	66
March	24	17	70	12	5	41
April	22	15	68	15	5	33
May	19	6	31	18	14	77
June	17	8	47	8	4	50
July	11	9	81	16	7	43
August	14	8	57	16	6	31
September	12	9	75	13	12	92
October	21	5	23	17	14	82
November	15	4	26	21	12	57
December	19	7	36	12	6	50
1967						
January	23	9	39	14	7	50
February	17	12	70	19	9	47
Total	227	118	(53)*	190	107	(55)*

*Average percent.

Table 2.—Detection of viruses in monkey kidney cell cultures (total: 86 lots)

Number of days after cells planted	Number showing virus infection			Percent showing virus infection		
	SV40	Foamy virus	TA*	SV40	Foamy virus	TA*
14–21	2	3	0	2.6	3.5	0
22–28	10	14	0	11.6	16.3	0
29–55	24	21	4	28.0	24.0	4.6

*TA = An unidentified agent.

days of conditioning before these monkeys were killed influenced the types of virus isolated; *i.e.*, a 30–90 day period of quarantine appeared to eliminate SV5 and measles virus infections. On the other hand, SV40 and foamy viruses appeared to persist for a prolonged period in the monkey kidney tissues.

The facts that SV40 enhances the growth of human adenovirus in monkey kidney cell cultures (*5*) and that adenovirus-SV40 hybrids may be obtained in monkey cell cultures infected with these viruses (*6*, *7*) lead one to believe that virus hybridization could occur with other mixed virus infections. Indeed, we have observed single cells doubly infected with SV40 and measles virus, SV40 and reovirus, and SV40 and foamy viruses. Nuclei doubly infected with SV40 and measles virus were observed under light and electron microscopy (*8*, *9*). Since the detection of latent viruses in cell cultures requires long-term cultivation of the cells, it is urged that precautions be taken whenever primary cell cultures are used for the production of virus vaccine to avoid contamination with adventitious latent viruses.

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GENERAL DISCUSSION

Dr. K. O. Smith: Dr. Hsiung, what was the type of monkey from which these cells were derived? Maybe I missed that. Was it an African green or rhesus monkey?

Dr. G. D. Hsiung: We used both rhesus monkeys and green monkeys, and almost 50% of each throughout the study.

Dr. R. J. Huebner: I would like to add a footnote to Dr. Melnick's definitions of latency. We have been struggling with the question of defective virus and have come up with a number of definitions of defective virus. One of them is a nondefective virus which happens to be defective only in the cell in which it is growing or in the host in which it is growing. Vogt, as you know, recently reported that defective NP cells—nonproducer chick cells—having defective sarcoma virus would yield infective virus if the right susceptible cell was found in some other chicken line. We found that the virus present in normal BALB/c mice will not grow in the cells of the BALB/c mouse but will grow in cells of the NIH Swiss mouse. On the other hand, a methylcholanthrene-induced tumor of the C57BL mouse contains leukemia virus which can be detected in cells of BALB/c mice but cannot grow in normal cells of C57BL mice.

So the question of latency and infectiveness—particularly important to vaccines—is whether or not you have a cell that is or isn't repressing its own virus particle probably through indigenous interferon.

Dr. K. Habel: Not only cultures from varied species but certainly the culture versus whole-animal *in vivo* infection, the conditions under which the cultures are maintained, all of these factors certainly influence relative susceptibilities. Therefore, they could be interpreted as evidence of defectiveness, which I think is the important point Dr. Huebner made.

Dr. W. McD. Hammon (Army Forces Epidemiological Board) : I have one question for Dr. Melnick and one for Dr. Hsiung.

Dr. Melnick, did I understand you to say that adeno-associated viruses types 1 through 3 were found only in man and not in monkeys? If so, I disagreee with that. But if I did misunderstand you, I apologize for having done so.

Dr. Hsiung, could you explain further what you meant by "TA" in your table 2?

Dr. J. Melnick: The data on which the statement was based are found primarily in the work of Dr. Hogan and his colleagues at the National Institutes of Health, in which they carried out antibody surveys in man for the four satellite or AAV types. Three of them were found to develop in man within the course of time very much like the adeno-antibody curves that Dr. Huebner reported at this Conference.

However, type 4 antibodies did not develop in the same way. Type 4 antibodies are present in high degree in green monkey species. As judged by the HI test that has been developed in our laboratory for type 4 satellite antibodies, similar findings were made: Green monkeys show a high frequency of antibody to type 4, and human beings do not.

Dr. Hammon: I'm afraid we still misundertand each other. What I interpreted from what you said was that monkeys were not infected with types 1, 2, and 3. Naturally we found antibodies repeatedly in monkeys with types 1, 2, and 3 and also isolated type 1 at least from monkeys.

Dr. W. P. Rowe: We found a great difference between the green and the rhesus monkey. The rhesus monkey has antibody to 1, 2, and 3, as is found in humans, whereas the green monkey has antibody only to 4. I think that is where the discrepancy is coming from. Our data fully agree with Dr. Hammon's.

Dr. Melnick: I stand corrected then. As far as humans are concerned, the data still hold that only 1, 2, and 3 antibodies have been shown.

Dr. Rowe: Right. Types 1, 2, and 3 in humans and in rhesus monkeys, and type 4 in green monkeys.

Dr. Melnick: Did you say there are no type 4 antibodies in rhesus monkeys?

Dr. Rowe: They are rare.

Dr. Melnick: The rhesus animals that come to Houston have many type 4 antibodies.

Dr. Habel: Dr. Hammon had another question: What does "TA" stand for?

Dr. Hsiung: "TA" is at present an unidentified agent. Thus far we know it is an ether-sensitive, medium-sized virus (RNA). In the classification we proposed earlier we think it is a new virus, but we cannot pin down exactly what virus group it belongs to. The name is just an initial of the person who did the work.

Dr. D. Franks: I'm not a virologist myself, and I would like to ask Dr. Melnick a question about the remark he made about the persistence of high levels of antibody to SV40 in children who were given poliovirus containing SV40 and his putting this forward as evidence for SV40 persisting as latent virus in these children. There are plenty of examples of nonreplicating antigens which can give rise to high levels of antibody in human beings, such as blood group antigen. Once you have antibody to blood group antigen, the antibody titer stays up for a long time, but you can't put this forward as evidence that blood group antigen is a latent virus.

Dr. Melnick: The work I referred to was that of Gerber carried out at the National Institutes of Health. As I recall his paper, he was working with sera from children who were vaccinated at the same time with a mixture of SV40 and inactivated polio vaccine. The SV40 antibodies persisted for 3 years. The polio antibodies declined. The polio vaccine in this case was an inactivated viral antigen. The SV40 was live. This would suggest that, when these two viruses are inoculated, the live one leads to persistent antibody and the inactivated ones does not.

Dr. Franks: The point I was trying to make about this is that whether antibody persists in high levels rather depends probably on how the individual handles the antigen. This was why I made the point about blood group antigen: that presumably there is something about the way certain antigens, for example, blood group polysaccharide antigens, are handled which gives rise to this persistence of high levels of antibody in contrast to other antigens, for example, proteins in which the titer can drop quite quickly. This might be the reason for the persistence of antibody and not that SV40 remained replicating.

Dr. Melnick: The types of antigen in the polio capsid and in the SV40 capsid seem to be similar to each other, since these are non-lipid-containing viruses. They both contain similar amounts of nucleic acid, although one is RNA and one DNA. I don't think the data Dr. Gerber obtained can settle the point. They just suggest something, in that the live virus gave rise to persistent antibodies in the same children whereas inactivated poliovirus did not.

Dr. T. Francis, Jr.: Just to continue this part of the discussion, Dr. Melnick, I think, stated there was no evidence that inactive virus or active virus used in vaccine persisted in the hosts, in the human host I gather. Is it not more correct to say this has not been studied with the possible exception he mentioned? That sounds as if you meant it really had been studied, and I don't believe it has.

Dr. Habel: I think the statement was that there is no evidence of latent infection established as a result of vaccination. But you're quite right. He should qualify this by saying "within the limited amount of looking that has been done," shall we say.

Dr. Francis: Reaction among children might be a very good source for looking at some of these things.

Dr. J. L. Bittle (Lederle Laboratories): Dr. Hsiung, how were your monkeys procured and isolated?

It seems to me the incidence of SV40 that you were reporting is extremely high, much higher than any the manufacturers found in polio vaccine production.

Dr. Hsiung: These monkeys were well conditioned. They were shipped directly from the field to New York and sent directly to the laboratory. The laboratory was worried about cross-contamination. Therefore, these monkeys were completely separated, rhesus monkeys on one side of the quarters, and green monkeys on the other side. Each monkey was caged individually. The caretaker was one person. This was an interesting part. One single person took care of all the animals. In the morning the caretaker took care of the green monkeys, then took a shower, cleaned up, and changed his clothes. Then he took care of the rhesus monkeys and went home. Theoretically, there was no, or minimum, cross-contamination.

In general, the green monkeys were highly susceptible to SV40. Even so, under such a strict separation, a cross-contamination occurred. Table 3 shows the cross-contamination between the two spaces.

Table 1 is a comparison of two studies. In 1964, the two species were kept in the same quarters. As you can see, virus infections in both species were almost the same. Apparently cross-contamination occurred. However, in 1966, the monkeys were completely separated as I described, and you can see here foamy agent occurred in the rhesus monkeys, whereas the SV40 occurred with much higher frequency in green monkeys.

Dr. Bittle: This is contrary to experience we had at Lederle Laboratories in well over 800 monkeys used for vaccine production. The incidence of SV40 was practically nil by testing by the standards of the Division of Biologics Standards, which do require long periods of incubation, up to 4–6 weeks, including two subcultures. So I still am a little perplexed by the high incidence of SV40 you found.

Dr. Hsiung: We were surprised too.

Dr. Habel: The geographical source of the monkeys in Africa might influence this. I don't know.

Dr. F. Abinanti: A notable example relative to latent infections following vaccination was the dramatic outbreak of scrapie, a chronic neurologic disease in sheep, which occurred in sheep vaccinated against louping ill. The vaccine was produced from formalin-inactivated brain material. Six or more months after the sheep received the vaccine, a significant number developed the neurologic signs and histopathology typical of the disease.

TABLE 1.—Comparative virus isolations from rhesus and African green monkeys

	1964		1966	
	Rhesus	Green	Rhesus	Green
Total number culture lots examined	64	34	187	157
Virus types isolated (%):				
SV5	25	30	0	0
Measles	12	3	0	0
Foamy agents	29	30	37	14
SV40	17	14	13	36
Mixed infection	25	30	6	3

Detection of Latent Viruses

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ONE of the main reservoirs of animal viruses in nature is in the form of agents occurring within the host without any evidence of their presence being observed or detected. Such a condition is referred to as a subclinical infection, and viruses that persist within the host cell, forming such a chronic inapparent type of hidden infection, without manifestation of disease are known as latent viruses. In the same vein, there are numerous instances of virus isolation from apparently undiseased individuals or animals by means of tissue culture. Earlier in this Conference, the viral flora and many latent viruses present in primate, nonprimate mammalian, as well as avian, tissue sources were discussed. On the other hand, latent viruses found in donor tissue as well as in recipients of vaccines have just been described.

The problem of latent or hidden viruses is a prominent one in relation to viral vaccines. With the increasing use of various tissue substrates of animal origin for propagation of virus for vaccine preparation, it is important to detect and assess presence of latent agents in donor tissues as a potential source of contaminants that could be associated with viral oncogenicity. As an example, simian virus 40 (SV40) was present as a latent agent in cultures derived from rhesus and cynomolgus monkey kidneys without evidence of infection, but produced marked cytopathic changes in kidney cultures from the heterologous grivet monkey. Furthermore, SV40 represents a simian latent virus that is an oncogenic agent, since it induces tumor formation in newborn hamsters. However, this same SV40 virus appears to be necessary for the growth of human adenoviruses in grivet monkey kidney cells and has led to reports concerning the possible hybridization of adenoviruses and SV40 (*1–4*). There is also the newly recognized group of small, defective viruses known as the "adenovirus-associated viruses" (AAV) that occur as latent agents and replicate only in cells infected with an adenovirus.

A phenomenon related to latent infections, developments of hybrids, and defective viruses is manifested at the cellular level in tissue culture as the carrier state. In fact, models of persistent infection in mammalian cells *in vitro* have been developed suggesting that the factor promoting the carrier state is interferon. Also, it has long been known that cell infection by some viruses suppresses or prevents further infection with other agents, and this phenomenon, known as interference, may also be used as a tool for detecting latent viruses in cell culture.

I shall restrict the discussion to summarizing some of the general techniques used for detecting latent viruses in cell cultures by immunological and interference procedures. Dr. Henle will discuss the topic of persistent infection and development of experimental models on the viral carrier state, while Dr. Rowe will cover problems on defective hybrid viruses.

IMMUNOLOGICAL TECHNIQUES FOR DETECTING LATENT VIRUSES

The more useful immunological tools for detecting persistent infection or latent viral protein in infected cells include fluorescent microscopy, diffusion methods, and complement-fixation tests.

Immunofluorescence is a qualitative test that has been used to identify cells in culture that are making virus protein and are presumably latently infected. The test is based on the fact that antibody labeled with a fluorescent dye, which may be specifically bound to a viral antigen, can be detected by its fluorescence under ultraviolet light (*5*). The main value of the technique has been in the localization of intracellular viral antigen. Also, examination of cells after infection may reveal the appearance of immunologically reactive protein and virus infectivity. By preparing antibody against different viral antigens selected from a spectrum of latent viruses frequently found in a particular host used to derive cells for culture, it is then possible to study and detect the appearance and presence of these various latent antigens during cell culture. The direct fluorescence method has the highest degree of specificity and is the procedure of choice for identifying viral agents in infected tissue, cells, exudates, and for studies on the development of latent viral antigens. The technique has its greatest application in the demonstration of latent viral antigens persisting in apparently normal tissue cells that are, in fact, infected.

Immunoprecipitation is a quantitative test wherein cell culture media or cell extracts suspected of containing a latent viral protein may be exposed to or reacted with an antiviral antibody previously obtained from sheep serum; this antigen-antibody complex then is precipitated with a second antibody against γ-globulin from sheep. This technique is useful in detecting minute traces of undetectable latent viral protein, providing a specific antiviral antibody is available for the reaction.

The complement-fixation test, a serological procedure, is best illustrated by the COFAL test for detecting the avian leukosis complex (*6*). The avian leukosis viruses are latent in the sense of only rarely manifesting their presence by producing a pathologic response in cultures of infected cells, and their importance lies in the fact that they are almost universally distributed among chickens. Suspensions of specimens to be assayed for the latent virus are inoculated into tissue cultures of secondary chicken-embryo fibroblasts. If sarcoma virus were present, typical transformation foci would be produced. However, if various leukosis viruses were present, neither cytopathic effect nor transformation would be observed, but CF antigens would develop. These CF antigens are demonstrable 10–20 days after inoculation by preparing concentrated cell pack suspensions of replicate infected cultures, which are then used as CF antigens in tests with a potent group-specific hamster antiserum prepared with the Schmidt-Ruppin strain of avian sarcoma virus. The test is simple to perform and provides specific information concerning the presence and immunologic relation of unknown latent viruses to the avian sarcoma-leukosis group of viruses.

In evaluating the various immunological methods for detecting viral proteins, two points must be kept in mind. 1) The antiserum is usually prepared by injecting previously isolated whole latent virus into an animal where the virus may or may not multiply. The antibody formed is against the surface protein of the whole particle. The immunologic reactivity of capsid protein as a part of an organized virus structure is not necessarily the same as the reactivity of an individual latent polypeptide chain of the capsid protein. In addition, to detect antigens of latent viruses which have several structural proteins, it is necessary to first separate the antigenic fractions of the known latent viruses so that standard specific antiserum can be made against each. 2) Proteins, formed under the direction of a latent virus genome, that do not become part of the virus particle or enzyme will not be detected by an antiserum against whole virus if the virus does not grow in the animal producing the antiserum. However, if the virus does multiply in the antiserum-producing animal, it is possible, but by no means certain, that the antiserum would have reactivity against all proteins specified by the viral genome.

INTERFERENCE TECHNIQUES FOR DETECTING LATENT VIRUSES

Another tool for detecting persistent or latent viruses makes use of various interference methods. In fact, some viruses are detectable in cell cultures only by interference or the reduced ability of the culture to support the growth of a challenged virus which produces a cytopathic effect. The test sample or virus is inoculated into cell cultures and after several days' incubation a known cytopathic virus is introduced into the same cultures. After further incubation, the titer of the cytopathic virus in the cultures is compared with that obtained in control cultures. Most viruses of the avian leukosis complex provide an excellent example, because they multiply in cultures of chicken embryo fibroblasts to high titer without causing any cytopathic change or cell transformation, even when most all the cells are actively producing virus. Replication of the leukosis virus can be detected in these cells by their failure to develop foci of infection when exposed to Rous sarcoma, a phenomenon termed "interference." The Rous sarcoma viruses consistently and frequently alter the infected chicken embryo cells. In fact, infection of normal chick-embryo cells with Rous sarcoma virus results in foci of transformed cells, the morphology of which may vary for the particular virus strain utilized. Virus activity is measured as the number of focus-forming units (FFU) per unit volume. The interfering property of the leukosis viruses is utilized to measure their activity in tissue culture in RIF (resistance-inducing factor) test. Cells chronically infected with leukosis viruses may be propagated in serial passage, yielding high titers on each transfer. This virus is capable of inducing neoplasia *in vivo* or of interfering with infection by Rous sarcoma virus *in vitro* and demonstrates the principle of a typical interference technique now used to detect latent viruses in cell culture. On the other hand, Somerson and Cook (*7*) report that growth of Rous sarcoma virus may be suppressed in tissue culture by a common latent tissue contaminant, *Mycoplasma orale.* The multiplication of Rous sarcoma virus and Rous-associated viruses was in fact inhibited in chick embryo fibroblasts infected with this tissue-culture propagated *Mycoplasma.* Hence, there is the demonstration of a latent agent (*Mycoplasma orale*) suppressing an interfering virus (Rous sarcoma) used to detect another latent agent (avian leukosis), thus giving a false-negative RIF test.

The converse of interference between viruses is exemplified by the condition in which certain latent agents acting as defective viruses are necessary for the replication of mature infectious virus. This has been demonstrated with AAV and adenoviruses, and more recently Smith and Gehle (*8*) demonstrated replication of an adeno-associated virus in both canine and human cells with canine infectious hepatitis virus serving as a helper. A similar observation has been made in our laboratories in which there is certain presumptive evidence that active infection with latent canine infectious hepatitis virus in canine lung cells may be initiated by acute serum from patients with infectious hepatitis. In these studies, the serum of a young woman with chronic hepatitis produced a cytopathic effect on canine lung cells and the agent isolated was identified as canine infectious hepatitis virus. This observation has been repeated with four serums from human hepatitis patients on three different lots of canine lung cells. At the same time, control canine lung cultures not inoculated with the human hepatitis serum showed no evidence of cytopathic effect or canine hepatitis virus. Hence, the question is raised whether the canine lung cultures carried a latent canine hepatitis virus or, perhaps, a canine-associated hepatitis virus that maturated to infectious virus in the canine cell system in the presence of human infectious hepatitis virus in the serum. If this observation is confirmed, it demonstrates the converse of the interference phenomenon and another role of a latent agent serving as a defective virus, but which could maturate to infectious virus under proper conditions.

SUMMARY

The extensive use of cell cultures for preparing and testing of virus vaccines in recent years has revealed a variety of latent agents that exist in the host tissues or that can be activated in some manner. Also, newer methods of virus isolation have vastly increased the number of latent viruses isolated from man. Such latent agents usually are inocuous at the cellular level and not detectable by a direct cytopathic effect. Also, the role of these latent viruses in human disease or tumorigenesis has not been established, and it has become necessary to develop specialized immunological techniques, interference methods, and other procedures for detecting their presence. Some of the general approaches more commonly used have been reviewed and problems in evaluating the results have been presented.

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Problems in the Detection of Viral Carrier States of Cell Cultures by Demonstration of Interference and Interferon[1]

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In certain viral carrier states of cell cultures the extent of the infection is kept under control by interference and interferon synthesis induced by the carried virus (*1*). In these instances the persistent infection may be detected by resistance of the cultures to superinfection by normally cytopathogenic viruses and by the demonstration of usually small amounts of interferon in the media. If such a type of carrier culture should be used inadvertently for preparation of viral vaccines, the production line most likely would come to an immediate halt because the yields of virus would be substandard. Not all persistent viral infections lead, however, to development of resistance and/or synthesis of interferon, nor does the detection of an interferon in spent media necessarily imply the existence of a viral carrier state.

These points can readily be illustrated by observations made with continuous suspension cultures of human cells derived from the hematopoietic system and composed of morphologically similar blast forms, that is, with cell lines derived from Burkitt's lymphomas, leukemic tissues, or buffy coats from the blood samples of patients with various diseases or of healthy donors (*2–10*). The data to be discussed merely serve as examples of problems that may have to be faced. They are not meant to suggest that any of these cell lines would ever be useful for vaccine production.

It was found that a small proportion of cells in some of these blast cell lines harbors a herpes-type virus, which represents an apparently new

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member of the herpes group (*3–8*, *11–13*). Among these carrier cultures, some indeed were found to be markedly resistant to vesicular stomatitis virus (VSV) and herpes simplex virus (HSV) and to produce low levels of an interferon. Others, however, were equally resistant, yet yielded no detectable amounts of interferon. Still others were highly susceptible to VSV and HSV but nevertheless synthesized interferon. Finally, one cell line was neither resistant to nor did it produce interferon. Similarly, among the cell lines known to be free of herpes-type virus, only two behaved as one would expect, in that they were highly susceptible to VSV and HSV and did not produce an interferon. Others, however, were equally susceptible yet yielded an interferon and, conversely, some showed resistance in the absence of interferon synthesis. No virus-free cell line was found to represent the fourth possibility, resistance and interferon production. It is obvious that in these examples neither assays for cellular resistance nor search for interferon activity provided dependable evidence for the presence or absence of the herpes-type viral carrier state, and that some of the blast cell cultures synthesized an apparently autogenous interferon, since it was produced without apparent stimulation (*14*, *15*).

To compound the situation further, exposure of these types of cultures to mumps virus led regularly to a persistent infection, whether or not the cell lines harbored the herpes-type virus (*16*). In both instances, practically all the cells became infected and produced mumps viral antigen(s); yet they continued to divide at nearly normal rates, giving rise to infected descendants. These results resemble those obtained by Walker and Hinze (*17*) upon infection of Chang's human conjunctiva cell line with mumps virus. The mumps viral carrier state abolished the resistance to VSV and HSV exhibited by some of the cultures. It did not *per se* induce interferon synthesis, but it failed to reduce autogenous interferon yields in the appropriate cell lines. In addition, it prevented interferon yields after stimulation by Newcastle disease virus and blocked the protective action of added human interferon (*16*). Thus, the mumps infection clearly reduced cellular resistance, apparently by inhibition of virus-induced interferon synthesis and action, in confirmation of observations made with members of the para-myxovirus group of viruses (*18–21*).

From these various results, it is evident that the herpes-type and/or mumps viral carrier states in some of these cell lines would escape detection if resistance to cytopathogenic viruses and interferon synthesis were the only available criteria. The single or dual carrier states were of course readily detected in these instances by appropriate immunofluorescence tests, since the viruses involved were known and sufficient viral antigen was produced in infected cells. These conditions are not regularly met in a search for viral carrier states in primary cell cultures and therefore other means for their detection will be required.

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Detection of Latent Viruses—Discussion

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ONE of the most serious problems in latent infections of tissue culture cells used for vaccine production is the unknown: the as yet undiscovered viruses that may be common in our culture systems or that may appear at some future date. This problem is particularly serious with monkey cells; due to the difficulty of experimental work with monkeys, our knowledge of their viral flora is probably far from complete. Also, we must not assume that the ecology of monkeys is stable: With the great geopolitical changes occurring in Africa and with changing methods of collecting, holding, and shipping of monkeys, we must anticipate appearance of new viral agents acquired from other species.

How can we monitor for viruses that we know nothing about? Of course there is no simple answer, but a fairly high degree of security could be obtained by a simple system of immunologic monitoring of cultures, using a procedure which Dr. Kendall O. Smith (personal communication) has employed for dog-cell cultures. Each lot of kidney cells could be planted in coverslip dishes at primary, secondary, and tertiary passage levels, and acetone-fixed for fluorescent antibody staining. These slips would then be stained by the indirect FA test with two types of sera, that of the tissue donor and a pool (or pools) of adult sera of the same species. Comparable testing should also be done with cell packs in the complement fixation test. Since most of the chronic viruses in kidneys represent coexistence of virus and antibody in the same host, this procedure should offer a broad spectrum of sensitivity. Almost certainly it would detect such known simian agents as SV40, SV5, salivary gland virus, reoviruses, adenoviruses, and probably B, foamy viruses, and measles as well. An additional test of this type should be the same type FA testing against coverslips infected with the vaccine virus; this at least would allow for detection of a new AAV serotype emerging in an adenovirus vaccine strain.

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By analogy with avian leukosis and LCM viruses, instances can be envisioned where the donor's serum would not react with the virus in his cells in culture, because of immunologic tolerance; however, a pool of adult sera from the same colony probably would.

In addition to prolonged cultivation, an important variable in obtaining growth of latent agents is serial cultivation; our clear impression is that foamy and salivary gland viruses emerge far more readily in secondary and tertiary cultures than in primary cultures, independently of the time of cultivation.

This type of approach, in conjunction with thin-section electron microscopy, could provide a much higher degree of security of freedom from indigenous viruses than obtained if one only looked for known agents.

GENERAL DISCUSSION

Dr. K. O. Smith: I would like to comment on Dr. Rowe's presentation. We have used this procedure to examine primary canine cells and simian cells. We now have some experience with the system suggested by Dr. Rowe. We used pooled γ-globulin from dogs. This is the total immunologic experience of about 1,500 dogs; we also used a battery of 5 sera, including the cell donor's serum. We used the indirect Coomb's reaction with fluorescein-conjugated anti-γ globulin.

In 33 lines from different street dogs which we examined, 26 contained at least one agent after 3–4 weeks' incubation, some of the cultures contained at least 2, and apropos of some of the comments that were made earlier, this system picks up many agents which produce no cytopathic effects. That is, less than 25% of our positive cultures showed any cytopathic effect at all.

We also have applied the system to simian cells, and it works well with the SV40 system. We are beginning a screening program to do essentially the same thing that Dr. Hsiung has done, using this sort of "universal antibody" system.

Dr. K. Habel: Did I understand you correctly that in dogs you did use pooled sera rather than viral specific donor antiserum?

Dr. Smith: We used pooled canine γ-globulin as a "universal reagent" and then used anti-canine γ-globulin conjugated with fluorescein. Also we used a battery of 4 street dog sera, which we found by experience had broad reactivity with known canine viruses, plus the dog's own serum.

Dr. H. C. Orr (Division of Biologics Standards): I don't know how many of you may be aware of a study that was made at Bionetic Research Laboratory. Dr. John C. Landon grew buffy-coat cells from the blood of chimpanzees, and these cells went into suspension. He became somewhat concerned that these cells might be like the human Burkitt cell system. He examined the cells by electron microscopy, and, sure enough, he found the typical Burkitt or herpes-like particle in them.

The point I want to raise here is that I was involved in the indirect fluorescent antibody test, examining serum from the autologous animal and from others. We indeed did get significant positive reactions showing, of course, that the autologous animal had specific antibodies in its serum.

But what I am wondering about specifically is whether the rhesus monkey system has been examined in this regard and, although one could not get suspension cultures from buffy coats of the rhesus or other monkeys, whether sera from such animals have been examined for the presence of this particular antibody.

Dr. P. Gerber: Yes, we did examine several species of nonhuman primates for antibodies to the herpes-like virus particles. Every single one, with the exception of baboons, had high titers of CF antibodies to the herpes-like virus particles. These studies have been published in the *Proceedings of the National Academy of Sciences*, vol. 58, 1967.

Dr. G. Brown (The University of Michigan): We have had some experience, too, with using the indirect method of immunofluorescence for checking each lot of cells that comes out of the monkeys. When we post the monkeys and prepare the tissue, we put some of the kidney tissue on coverslip cultures. Just as soon as they are laid down we test with the serum from the monkey itself.

We found a very high percentage of correlations with adventitious agents in the monkeys. Actually we were using green monkeys in the summer of 1967, and did not find very many; then with the scare from Germany we shifted over to a newly arrived lot of cynomolgus monkeys and every single one was just loaded with these agents.

As a matter of fact, we haven't identified all of them, but I am confident that there were several viruses present.

The point is the FA is a procedure which gives you the tip-off on this even before the cells are ready to be used in the laboratory and you are really forewarned. We are just now turning to rhesus, and we don't have any data on the rhesus yet.

Dr. Habel: Does anybody have any comment to make concerning possible isoantigen differences between noninbred animals and possible nonviral causes for such staining?

Dr. D. Franks: This is the very point I wanted to make. Of course, if you use the same animal from which you got the cultures, you will get over this difficulty about isoantigens, but if you use pools of sera from adult animals, then you might run into this problem.

The extent to which isoantibodies in adult sera which can react with tissue cells of other individuals will complicate these investigations is difficult to assess. However, isoantibodies for human leukocyte antigens are frequently found, particularly in multiparous women or in patients who have received multiple transfusions. These antibodies can react with some human cell lines, but not with other cell lines (Metzgar and Danagan 1965, *Histocompatability Testing,* NAS–NRC Publication 1229, p. 183; Franks and Gunner, *Experimental Cell Research,* vol. 37, p. 693, 1965).

A further comment that I would like to make is that, although I am sure this technique can detect viruses in the cells, there are two difficulties:

One is that you are looking for viruses that you are not sure are there. It seems to me that it is a retrograde step to go away from the use of specific antibodies that you know something about.

The other difficulty is that you could get positive fluorescence for several other reasons. For example, one wonders what things, like rheumatoid factor, would do in this sort of system. After all, these cells are grown in sera from other species in which there may be antibodies to those cells, and the antibodies may be fixed on the cells. If then, you add serum, even from the same animal that provided the cells, and if this animal has a rheumatoid factor in it, the rheumatoid factor would presumably be fixed on the cells, and you could pick this up with a fluorescent antibody. I would like to put that forward as a note of warning about using this sort of technique.

Dr. Habel: At least in the case of the use of autologous serum, it would show an abnormal situation concerning the presence of an antigen in the animal's cells and his own serum having antibodies against it; perhaps this is not the kind of cell we want to use for vaccine production.

Dr. Smith: We have had some experience along these lines in trying to determine whether this was a prime factor or not. I can relate three experiences.

One is that some of these preparations can be frozen, thawed, and sonic-oscillated, and cell-free materials can be passed.

Secondly, some of these, or most of these, show foci which on prolonged incubation will grow. The fluorescing foci become larger, suggesting an infectious process.

Finally, we have done as Dr. Franks has suggested. We have gotten together a battery of antisera against known canine agents and some of these isolates fall out with a known antiserum.

Dr. P. A. Long (U.S. Department of Agriculture, Regional Poultry Research Laboratory, East Lansing, Mich.): Dr. Rightsel made three statements on RIF and COFAL tests. Most people here I feel will agree that these two tests are not unequivocal in result.

In my hands I have seen that RIF viruses or the helper viruses of Rous sarcoma of the B subgroup do show a distinct cytopathic effect. So they are not latent in the form that they do not show their presence.

In addition, we have an agent which activates Rous NP cells of chick embryo origin but are COFAL-negative and RIF-inactive. We also have an agent isolated from our inbred 15–I flock which does not show any clinical disease manifestation of tumor. It also activated NP cells. It shows RIF activity and it is COFAL-positive. So you cannot negate systems using any one system for these viruses.

Dr. W. H. Murphy: Dr. Melnick, would you comment on the distribution of herpes-type viruses in human leukocytes. From what I can remember, it seems that Moore's group and Grace's group have detected them in cells, but the Harvard group, Dmochowski's group, and Murphy's group have not. I don't think Melnick has reported on herpes-type viruses in leukocyte cultures. I don't have adequate information. Are these viruses found most commonly in Burkitt-derived cells? Also, how much work has been done to demonstrate the retrieval, or what references are there to demonstrate the retrieval of leukemogenic viruses of C-type virus particles from dog lymphomas? Perhaps I didn't understand your statements.

Dr. J. Melnick: I don't think the last word has been said on the incidence of recovery of the herpes-type virus particles from different cultures that have been derived from peripheral blood, leukocytes, or from bone marrow cells. The results vary somewhat in the different laboratories, and maybe they depend on the conditions under which the cells are cultivated or on what else is being propagated in the laboratory. Some laboratories have reported a very high recovery rate. Our own laboratory finds a very low rate.

The fact that the antibody to this virus is so widespread should not make it surprising that the virus should be recovered from time to time, but the optimal conditions of its recovery are still to be worked out.

In regard to your question about the recovery of viruses from dog lymphoma, this is something that is being studied at the moment, but I don't know that there has been any success in this regard. Dog lymphosarcoma cells have been passed, but nobody has shown any virus in these cells.

A new approach would be to use the serum of the dog with a lymphoma in the same way that Dr. Huebner used the hamster carrying Schmidt-Ruppin-Rous sarcoma virus. Such a serum may be able to detect antisera for the group-specific antigen for dog leukosis viruses if they exist.

Dr. Habel: Dr. Henle, do you want to comment on the ubiquity of herpes?

Dr. W. Henle: No.

Dr. T. Francis, Jr.: I'm just reminded of an interesting thing that might be of some value to this consideration. Years ago when the use of the hot box was being discussed for treatment of malaria and also treatment of syphilis, Stafford Warren reported frequent occurrence of herpes in monkeys—which was thought not to occur. One wonders if that might be another way of trying to elicit some of these so-called latent infections or at least give some indication of them.

Dr. R. Haff (Smith, Kline & French) : Getting back to the indirect fluorescent test using homologous serum, has anyone attempted to use this method to seek unknown agents in human embryonic cells?

Dr. Habel: Does anyone have any experience with the use of pooled γ-globulin—I assume you referred to pooled human γ-globulin—being used in the indirect FA staining of human cell cultures?

Dr. Smith: We have had limited experience with this but not with primary cells. We have used only stable cell lines. We have picked up fluorescence in several cell lines, but we haven't done the necessary work to determine whether this is some sort of a cell reaction or whether it is due to an infectious agent.

Dr. Habel: I would like to remind everyone that with long continued culture of any cell line in a laboratory, you increase the chances of accidentally introducing agents that happen to be in this environment. Certainly this must be kept in mind, particularly in experimental laboratories (as compared to production laboratories) where the usual techniques and precautions are not being taken to have one area used for only one agent.

Detection of Viral Latency by Biochemical Methods

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I will limit my discussion to those phenomena where virus replication is blocked, reversibly or irreversibly, at some intermediate stage of the virus growth cycle. Virus-cell interactions of this type result either from infection with "defective" or "incomplete" virus, or from the inability of a particular cell type to support the complete replicative cycle of a specific virus type. Included here is the type of latency of special importance to this Conference, "viral transformation" (*1*). The ease with which biochemical methods, especially DNA–RNA, DNA–DNA, and RNA–RNA homology measurements, can detect latent virus infections depends on the phase of the virus growth cycle affected. Many steps in DNA virus replication are reasonably well characterized (*2*), and methods are available for the analysis of the viral intermediates present at these steps. Thus, possible latent infection by DNA viruses often may be successfully detected biochemically. Much less is known about the RNA virus types important for this Conference, such as the avian sarcoma virus and the murine and avian leukemia viruses. Biochemical studies of virus-cell systems using these RNA viruses are needed to provide the necessary basic information and methodology for the biochemical detection of latent infection by these viruses.

I will describe briefly the phases of the virus growth cycle where a block in virus replication possibly may occur and the available biochemical methods for detecting viral components present at these interrupted stages of virus development. Very few "latent virus" infections have been analyzed biochemically, and this discussion concerns mainly ways and means of detecting hypothetical latent infections by known animal viruses, of which we now recognize over 500 (*3*). A serious difficulty is that infection may be caused by viruses not yet discovered whose presence may be difficult to detect.

DNA VIRUSES

The establishment of viral latency might be initiated at any step of viral replication: 1) after the uncoating of parental viral DNA, 2) after the synthesis of early viral mRNA, 3) after the replication of viral DNA, 4) after the synthesis of late viral mRNA, 5) in the translation of viral mRNA molecules, or 6) in the final assembly of the virion. Biochemical procedures can be used to detect viral intermediates at the various stages of virus development, as described below.

Detection of Parental Viral DNA

The detection of a few viral DNA molecules in an infected cell is a formidable task technically, since only a small portion of the total DNA of the cell would be represented by viral DNA. For example, a single viral gene in a mammalian cell would represent only one part of viral DNA in 10 million parts of cell DNA, a whole papovavirus genome one part in 2 million, and the larger adenovirus genome one part in 300,000.

Cells transformed by DNA viruses appear to contain a small number of copies of the viral genome; replication of viral DNA, if it occurs, is probably limited to the synthesis of, at most, 10–50 copies. Studies attempting to detect viral DNA in cells transformed by polyoma virus, SV40, and adenovirus by homology measurements between DNA from transformed cells and viral DNA or synthetic complementary viral RNA have not yielded conclusive results (*2*). For this type of experiment it is crucial that viral DNA preparations contain no cell DNA. The presence of cell DNA in polyoma DNA preparations used as reagents for this analysis, due to the incorporation of cell DNA into the virion during replication (*4*, *5*) further complicates the interpretation of the results of some of these experiments.

An attempt to detect homology between the DNA from virus-free adenovirus (Ad) type-12 tumor cells and Ad 12 synthetic complementary RNA (Ad 12 cRNA) is shown in table 1. Ad 12 cRNA binds to a higher extent with DNA from the cell transformed by Ad 12 than with DNA from polyoma tumor cells or normal hamster cells, suggesting the presence of Ad 12 DNA in cells transformed by Ad 12. Ad 2 cRNA binds to a lower extent to cell DNA's. The low binding of both Ad 2 cRNA and Ad 12 cRNA to normal cell DNA adds uncertainty to the interpretation of these data. Such binding may possibly be due to 1) true hybrid formation between nucleotide sequences common to both virus and cell, 2) mismatched association between DNA segments, or 3) the possible presence of small amounts of cell DNA in viral DNA preparations used as templates for the synthesis of Ad 12 cRNA. Further refinements in the hybridization reaction are needed to detect convincingly small amounts of viral DNA in the presence of large amounts of cellular DNA.

TABLE 1 (*6*).—Hybridization of Ad 12 complementary RNA with Ad 12 tumor DNA

Expt. No.	Source of DNA[a]	Complementary RNA bound (cpm)[b]		
		Ad 12	Ad 2	*Pseudosphaeriaceae aeruginosa*
1	Ad 12 hamster tumor cells	166[c]	77[d]	—
	Polyoma hamster tumor cells	40	61	—
	Salmon sperm	23	44	—
	Ad 12 virus	3821	66	—
	Ad 2 virus	54	3609	—
2	Ad 12 hamster tumor cells	117[e] (2)[f]	65[g] (2)	6[h] (2)
	Baby hamster kidney cells	51	54	0
3	Ad 12 hamster tumor cells	93[i] (2)	48[j] (2)	27[k] (2)
	Salmon sperm	60 (2)	48	31 (2)
4	Ad 12 hamster tumor cells	142 (2)[l]	68 (2)[m]	—
	Ad 12 hamster tumor cells	114 (2)[n]	54 (2)[o]	—
5	Ad 12 hamster embryo cells[p]	105[q]	62[r]	—
6[s]	Ad 12 hamster tumor cells	388[t]	31[u]	—
	Ad 12 hamster tumor cells	389[v]	122[w]	—

[a] 100 μg DNA fixed on each filter and incubation 14 hours at 66°C for expts. 1–5; [b] background (cpm bound to empty membrane) subtracted, usually from 7–20 cpm, and RNA made on denatured DNA template in expts.; 1–5; [c] 18,500 cpm/ml input; [d] 18,800 cpm/ml input; [e] 7530 cpm/ml input; [f] number of separate reactions; [g] 7950 cpm input; [h] 8700 cpm input; [i] 7920 cpm input; [j] 9600 cpm input; [k] 10,400 cpm input; [l] 8650 cpm input; [m] 9140 cpm input; [n] 11,600 cpm input; [o] 10,100 cpm input; [p] 50 μg only on membrane and incubated 40 hours at 66°C; [q] 11,900 cpm input; [r] 11,100 cpm input; [s] 72-hour incubation; [t] 11,760 cpm input; [u] 9480 cpm input; [v] 10,180 cpm input and cRNA made on native DNA template; [w] 8880 cpm input and cRNA made on native DNA template.

Detection of "Early mRNA"

If the parental viral genome is transcribed, the demonstration of virus involvement is easier. Early mRNA, *i.e.*, mRNA transcribed before viral DNA synthesis begins, has been demonstrated in cells infected by several DNA viruses, including Ad 2 (*7*), vaccinia virus (*8*), and polyoma virus (*9*) by DNA–RNA hybrid formation between labeled RNA from infected cells and viral DNA. For example, ^{3}H-uridine-labeled RNA from the cytoplasmic polyribosomes, 6 hours after infection with Ad 2, was annealed with Ad 2 DNA (table 2). About 2% of this RNA hybridized specifically with Ad 2 DNA, thus demonstrating the presence of early Ad 2 mRNA. [Ad 2 DNA replication starts about 7 hours after infection (*10*).]

Virus-transformed cells may represent a natural situation in which only early mRNA is synthesized. As depicted in text-figure 1, viral genes, incorporated into the cell during transformation, may be transcribed to form viral mRNA molecules in the cell nucleus. Viral mRNA molecules are subsequently transported to the cytoplasm to form viral polyribosomes. To test for viral mRNA, first, radioactive RNA is isolated from the nucleus or polyribosomes of transformed cells after growth in medium containing radioactive precursors of RNA, such as ^{3}H-uridine or ^{32}P-inorganic phosphate. Second, viral DNA is isolated from purified virus and extensively purified. Third, labeled RNA and viral DNA are annealed, with the hybridization technique described by Gillespie and Spiegelman (*11*). The formation of DNA–RNA hybrids provides direct evidence for the presence of viral mRNA.

Table 2 (*7*).—Hybridization of pulse-labeled RNA from polyribosomes of adenovirus 2-infected KB cells with viral DNA *

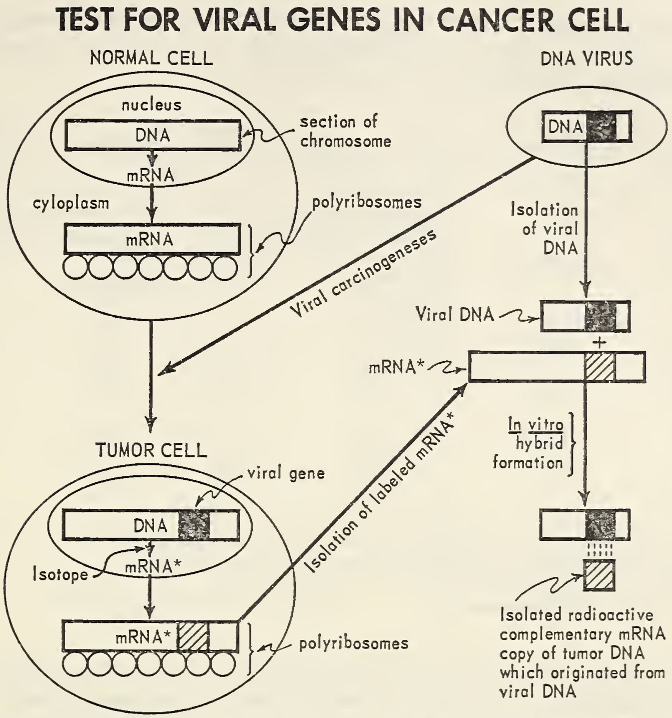
Source of RNA (hr after infection)	Input RNA (cpm)	Adenovirus DNA (µg/filter)	Bound RNA (cpm)	Bound (%)
Uninfected cells	3250	3	11	0.34
(6-hr control)	3250	3	11	0.34
	3250	None	9	0.28
Infected cells	3440	3	50	1.5
(6 hr)	3440	3	58	1.7
	3440	None	3	0.09
Infected cells	2650	3	446	16.8
(12 hr)	2650	3	425	16.1
	2650	None	19	0.9
Infected cells	2260	3	706	31.2
(18 hr)	2260	3	721	31.9
	2260	None	22	0.9
Infected cells	1950	3	204	10.5
(24 hr)	1950	3	214	11.1
	1950	None	18	0.9
Uninfected cells	3275	3	19	0.58
(24-hr control)	3275	3	17	0.52
	3275	None	18	0.55
Synthetic "complementary"	2840	3	954	33.6
RNA†	2840	3	984	34.7
	2840	None	2	0.07

* The entire experiment, including all time intervals, was repeated with similar results. The 18-hour point was further repeated twice.

† Made on an Ad 2 DNA template using purified RNA polymerase kindly supplied by Dr. S. B. Weiss.

An example of this analysis is shown in table 3. Viral-specific mRNA is readily detected in Ad 12-transformed cells by annealing radioactive nuclear or polyribosomal RNA with Ad 12 DNA (*12*). With 52,000 cpm of input polyribosomal RNA, 130–140 cpm were specifically hybridized to Ad 12 DNA, only 2 cpm binding to an empty membrane. With 214,000 cpm of input nuclear RNA, 440 counts were hybridized, only 26 cpm binding to an empty membrane. In control experiments, radioactive RNA isolated from normal human and hamster cells, or cells transformed by polyoma or SV40, did not hybridize with Ad 12 DNA.

Large quantities of viral-specific mRNA are detected readily both in the nucleus and polyribosomes of tumor cells induced by seven different adenoviruses, both "highly" and "weakly" oncogenic (*12*, *13*) (table 4). Of the RNA prepared by labeling transformed cells for 3 hours, 6–27 parts per 10,000 are viral specific. Two distinctly different populations of viral mRNA molecules are recognized by comparative hybridization analyses, one specific for tumor cells induced by "highly" oncogenic adenoviruses and the other specific for tumor cells induced by "weakly" oncogenic adenoviruses. The sensitivity of this method can be further in-


TEST FOR VIRAL GENES IN CANCER CELL
NORMAL CELL
DNA VIRUS
nucleus
DNA
section of chromosome
mRNA
cytoplasm
polyribosomes
mRNA
DNA
Isolation of viral DNA
Viral carcinogeneses
Viral DNA
+
mRNA*
In vitro hybrid formation
Isolation of labeled mRNA*
TUMOR CELL
viral gene
DNA
Isotope
mRNA*
mRNA*
polyribosomes
Isolated radioactive complementary mRNA copy of tumor DNA which originated from viral DNA

TEXT-FIGURE 1.—This scheme depicts incorporation and transcription of viral genes in cells neoplastically transformed by virus infection. Viral mRNA synthesized in the cell nucleus is subsequently transported to the cytoplasm where viral polyribosomes are formed. Cancer cells are grown in medium containing radioactive precursors of RNA. Labeled RNA from the nucleus and polyribosomes is isolated and annealed with viral DNA isolated from purified virus. The formation of viral DNA-labeled RNA hybrids provides direct evidence for the presence of functioning viral genes in the cancer cell.

TABLE 3 (*12*).—Hybridization of RNA from polyribosomes and nuclei of AD 12-transformed cells with viral DNA

RNA from	Input RNA (cpm)	AD 12 DNA μg/filter	Bound RNA (cpm)	Bound % (background not subtracted)
Polyribosomes*	52, 440	3 μg	146	0. 28
	“	3 μg	134	. 26
	“	None	2	. 004
Nuclei*	214, 000	3 μg	438	. 20
	“	3 μg	423	. 20
	“	None	26	. 01
	42, 450	3 μg	97	. 23
	“	3 μg	114	. 27
	“	None	12	. 03
Whole cell*	283, 100	3 μg	356	. 13
	“	3 μg	406	. 14
	“	None	60	. 02
	59, 600	3 μg	113	. 19
	“	3 μg	103	. 17
	”	None	14	. 02

*Labeled during 180-minute period with ^{3}H-uridine (4 μc/ml).

creased by using shorter labeling times and annealing polyribosomal RNA with viral DNA. Under such conditions, up to 2% of the polyribosome mRNA fraction hybridizes with viral DNA (*14*).

TABLE 4 (*15*).—Presence of viral specific RNA's in tumor cells induced by adenoviruses

Inducing virus	Oncogenicity	Polyribosomal RNA* fraction bound (× 10^4)‡		Nuclear RNA† fraction bound (× 10^4)‡	
		Homologous DNA	None§	Homologous DNA	None
Ad 12	High	27.0	0.4	16.2	0.6
Ad 18	"	6.6	1.5	8.0	0.7
Ad 31	"	7.7	1.7	8.1	1.4
Ad 3	Weak	8.6	1.8	6.3	1.5
Ad 7	"	9.4	1.2	10.8	0.7
Ad 14	"	12.8	1.2	6.6	0.8
Ad 16	"	8.0	1.2	14.4	0.9

* Polysomal RNA from tumor cells labeled for 180 minutes with ^{3}H-uridine (4 μc/ml).

† Nuclear RNA from tumor cells labeled for 180 minutes with ^{3}H-uridine (4 μc/ml).

‡ Average of duplicate hybridization reactions.

§ Fraction bound to empty membrane.

Recently Freeman *et al.* (*16*) have transformed rat embryo cells *in vitro* with "nononcogenic" Ad 1, 2, 5, and 6. Such cells have altered growth properties and morphology, but they have not yet been shown to be neoplastic. As shown in table 5, Ad 2 mRNA is readily detected in these cells (*15*). Ad 2 mRNA hybridized with Ad 1, 2, 5, and 6 DNA, but not with the DNA of either the "weakly" oncogenic adenoviruses such as Ad 7 or the "highly" oncogenic adenoviruses such as Ad 12. Therefore, three different groups of viral-specific mRNA molecules were synthesized by human adenovirus-transformed cells, one specific for the "highly" oncogenic group, Ad 12, 18, and 31, the second specific for the "weakly" oncogenic group, Ad 3, 7, 11, 14, 16, and 21, and the third specific for the Ad 1, 2, 5, and 6 group.

Benjamin (*9*) has demonstrated small quantities of viral-specific RNA in cells transformed by the small DNA viruses, SV40 and polyoma. Thus cells transformed by any of the DNA viruses probably synthesize sufficient quantities of viral-specific RNA to be detected by DNA–RNA hybrid formation with viral DNA.

The DNA–RNA hybridization technique is extremely sensitive and specific and may be used for evidence that cells are not transformed or latently infected by a specific virus. For example, as shown in table 6, tumor cells derived from hamsters inoculated with Ad 10 do not contain detectable Ad 10-specific mRNA; tumor tissue from hamsters inoculated with Ad 11 do not contain detectable Ad 11 mRNA, and tumor cells from a hamster inoculated with an adenovirus isolate of unknown type do not possess mRNA molecules specific for Ad 2, 16, or 12. Therefore, we conclude that

TABLE 5(*15*).—Hybridization of ^{3}H-RNA from Ad 2-transformed cells with different adenovirus DNA's

RNA input*	DNA (3 μg/filter)	cpm bound	Background subtracted	
			% bound	% relatedness
303,000 cpm	Ad 1	223 212	0.070	102
	Ad 2	216 211	.069	100
	Ad 4	19 18	.004	<10
	Ad 5	202 205	.065	94
	Ad 6	217 210	.069	100
	Ad 7	24 27	.006	<10
	Ad 8	26 25	.006	<10
	Ad 12	26 22	.006	<10
	Ad 26	18 14	.003	<10
	SV40	12 13	.002	<10
	Pseudosphaeriaceae aeruginosa	9 11	.001	<10
	None	9 5	—	—

*RNA extracted by hot phenol-SDS procedure from Ad 2-transformed rat embryo cells after exposure to ^{3}H-uridine (4 μc/ml) for 180 minutes.

TABLE 6 (*15*).—Absence of adenovirus-specific RNA from tumors of hamsters inoculated with Ad 10, Ad 11, and an unknown isolate

RNA input cpm	DNA 3 μg/filter	Bound cpm	% bound	Background subtracted	
				Bound cpm	% bound
Tumor cell line from hamster inoculated with Ad 10. Whole-cell RNA, 3-hr labeling, 235,400 cpm.	Ad 10	27	0.011	12	<0.010
	Ad 10	21	.009	6	<.010
	None	15	.006	—	
Tumor tissue from hamster inoculated with Ad 11. Whole-cell RNA, 16.5-hr labeling, 60,100 cpm.	Ad 11	5	.008	—	<.010
	None	8	.013	—	
#H-3557 tumor cell from unknown Ad-inoculated hamster,* whole-cell RNA, 3-hr labeling 61,100 cpm.	Ad 12A	5	.008	—	<.010
	Ad 12A	6	.010	—	
	Ad 16	7	.011		<.010
	Ad 16	2	.003	—	
	Ad 2	3	.005	—	<.010
	Ad 2	1	.002	—	
	None	4	.007	—	
	None	8	.013	—	

*Unknown adenovirus type isolated from child.

these three tumors were most likely not caused by the adenoviruses used to inoculate these animals and probably arose spontaneously. In another example, rat embryo cells exposed to Ad 4 by Dr. A. Freeman were somewhat morphologically altered, but did not behave like typically adenovirus-transformed cells. As shown in table 7, these cells did not synthesize detectable amounts of Ad 4 mRNA. However, one word of caution: The possibility must be considered that these cells are virus transformed but synthesize too little viral mRNA to be detected by hybridization.

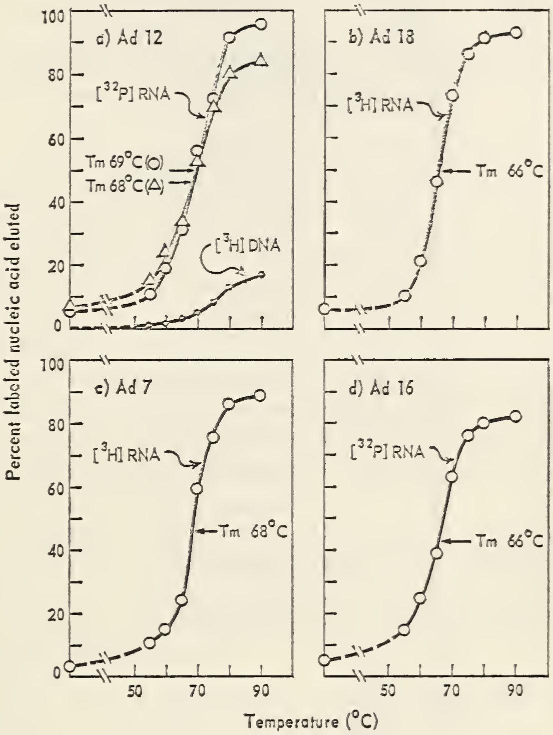
The thermal denaturation profiles of the DNA–RNA hybrids formed between adenovirus DNA's and the purified Ad 12, 18, 7, and 16 mRNA's are shown in text-figure 2. Increasing the temperature causes dissociation of the DNA–RNA hybrids. The midpoint of the denaturation profile is defined as Tm values. These values range from 66–69°C, indicating base compositions of 47–48% G + C for the adenovirus mRNA's made in transformed cells. We directly analyzed the ^{32}P base composition of purified adenovirus mRNA preparations, as shown in table 8. The base composition of the viral mRNA's is distinctly different from normal cell RNA components (ribosomal RNA, transfer RNA, and nuclear RNA) with regard both to base ratios and average G + C content. The G + C contents of the Ad 12, 18, 7, and 16 mRNA's are 47–48%, the content of Ad 2 mRNA is 49%, and that of Ad 5 mRNA is 50% G + C. Although the G + C contents of Ad 12 and 18 mRNA's do not differ significantly from those of their

TABLE 7 (*15*).—Hybridization of Ad 4 tumor cell (?) RNA with different adenovirus DNA's

RNA input*	DNA 3 μg/filter	Bound cpm	Background subtracted	
			Bound cpm	Bound %
309,800	Ad 2	25 23	10	0.0032
	Ad 4	16 19	4	.013
	Ad 6	20 23	8	.0026
	Ad 7	25 23	10	.0032
	Ad 8	24 21	9	.0029
	Ad 12	23 23	9	.0020
	Ad 16	23 20	8	.0026
	Ad 18	18 21	6	.0019
	Ad 26	24 25	11	.0036
	None	17 10	—	—

*Whole-cell RNA extracted from Ad 4 tumor cells (?) and purified by hot phenol SDS treatment without the use of deoxyribonuclease.

viral DNA's, the G + C content of Ad 7 and 16 mRNA's is 2–5% lower than the contents of their DNA's, which are 50–52% G + C. The Ad 2 and 5 mRNA's isolated from transformed cells contain 49 and 50% G + C, while the DNA of these viruses contain 56 and 58% G + C, respectively. These results indicate that viral DNA regions with 47–50% G + C are incorporated or preferentially transcribed in adenovirus-transformed cells.



TEXT-FIGURE 2.—Thermal elution profile of labeled nucleic acids from membrane filter containing DNA–RNA hybrids formed between *a*) Ad 12 ^{3}H-DNA (○) and Ad 12-specific ^{32}P-RNA (○) purified by one cycle of hybrid formation and elution; Ad 12 DNA and Ad 12-transformed cell nuclear ^{32}P-RNA (△), *b*) Ad 18 DNA and Ad 18-specific ^{3}H-RNA purified by one cycle of hybrid formation and elution, *c*) Ad 7 DNA and Ad 7 tumor cell nuclear ^{3}H-RNA, and *d*) Ad 16 DNA and Ad 16-specific ^{32}P-RNA purified by one cycle of hybrid formation and elution.

Detection of Progeny Viral DNA

Replicating viral DNA is readily detected in the infected cell by several procedures. DNA–DNA hybrid formation between labeled DNA from infected cells and viral DNA is reliable and generally applicable. As shown in table 9 (*6*) Ad 2 DNA is detected readily in Ad 2-infected KB cells by labeling the total DNA of the cell with ^{3}H-thymidine and determining if homology exists between labeled replicating DNA and Ad 2 DNA.

Table 8.—Base composition of adenovirus-specific RNA isolated from tumors and transformed cells

	Number of determinations	Mole % viral-specific mRNA*				G + C	Viral DNA† G + C
		C	A	G	U		
Ad 12-transformed hamster embryo cell...	4	19. 1 ± 1. 4	25. 8 ± 1. 4	29. 0 ± 1. 3	26. 1 ± 1. 3	48. 1 ± 0. 7	49
Ad 18-induced hamster tumor cell..........	4	18. 5 ± 0. 6	24. 6 ± 1. 4	29. 6 ± 0. 7	27. 4 ± 0. 8	48. 1 ± 1. 1	48
Ad 7-induced hamster tumor cell...........	3	19. 9 ± 0. 8	25. 6 ± 0. 4	27. 5 ± 0. 5	27. 0 ± 0. 2	47. 4 ± 0. 4	51
Ad 16-induced hamster tumor cell..........	9	22. 1 ± 0. 9	23. 9 ± 1. 2	25. 4 ± 0. 9	28. 7 ± 1. 2	47. 5 ± 0. 5	52
Ad 2-transformed rat embryo cell..........	8	23. 1 ± 0. 4	24. 4 ± 0. 6	25. 7 ± 0. 3	26. 9 ± 0. 5	48. 8 ± 0. 4	56
Ad 5-transformed rat embryo cell..........	4	22. 1 ± 1. 1	23. 2 ± 0. 5	28. 4 ± 0. 9	26. 3 ± 0. 4	50. 6 ± 0. 7	58

*Fujinaga and Green (*12*).

†Piñna and Green (*17*).

TABLE 9 (*6*).—Detection of replicating viral DNA in AD 2-infected KB cells

Membrane	Solution*		Bound DNA	
	DNA	cpm	cpm	% of input
Ad 2 DNA	Ad 2 KB, 18 hrs	407	363	89
3 μg/filter	Ad 2 KB, 18 hrs	407	372	91
	Ad 2 KB, 24 hrs	444	395	89
	Ad 2 KB, 24 hrs	444	405	92
	Ad 2 DNA	454	430	95
	Ad 2 DNA	454	436	96

*Ad 2-infected KB cell suspensions were labeled with ^{3}H-thymidine for 3 hours at 18 and 24 hours after infection. Total cell plus viral DNA was isolated and annealed with Ad 2 DNA.

Replicating viral DNA also may be detected by taking advantage of differences in physical and chemical properties between virus and cell DNA, such as buoyant density, size, and circularity. For example, pseudorabies DNA (*18*) is recognized by its much higher buoyant density than cellular DNA. Likewise, Ad 2 DNA is readily detected in infected cells by density gradient centrifugation (*19*). DNA isolated from cells after labeling with ^{3}H-thymidine at different times after infection with Ad 2 was centrifuged to equilibrium in CsCl density gradients with marker Ad 2 ^{32}P-DNA. As shown in text figure 3, Ad 2 DNA is recognized by the ^{3}H-labeled DNA coinciding with the marker Ad 2 ^{32}P-DNA. Rapp, Feldman, and Mandel (*20*) have demonstrated, similarly, the synthesis of viral DNA in monkey kidney cells abortively infected with Ad 2.

The presence of replicating polyoma DNA (*21*) and SV40 DNA (*22*) in infected cells can be recognized by heat denaturation of the DNA preparations and subsequent chromatographic separation of the denatured cellular DNA from denaturation-resistant circular viral DNA on methylated albumin and nitrocellulose columns.

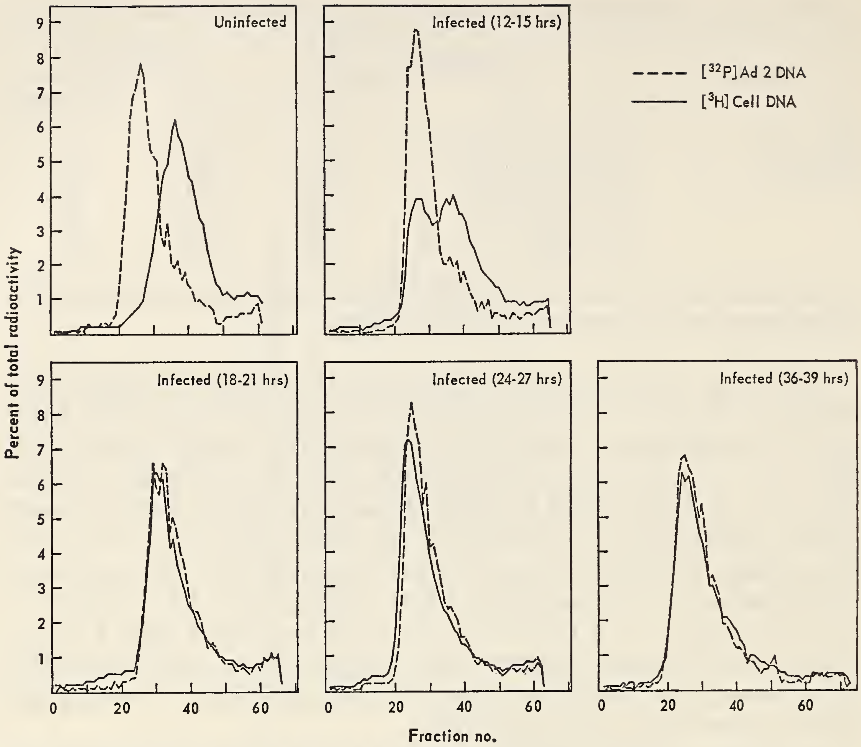
Other methods, not yet exploited, may take advantage of the higher molecular weight of poxvirus and herpesvirus DNA's as compared to cellular DNA (as isolated by standard procedures).

Detection of Late mRNA

Large amounts of late viral-specific mRNA, *i.e.*, mRNA transcribed after initiation of viral DNA synthesis, are synthesized by virus-infected cells. At least 30% of the labeled RNA isolated from the polyribosomes of Ad 2-infected cells is virus specific (*7*) (table 2). Large amounts of late mRNA are synthesized also in vaccinia virus-infected cells (*8*).

Synthesis of Viral-Specific Proteins

All DNA viruses studied so far induce the synthesis of increased amounts of several enzymes involved in DNA synthesis. The analysis of enzyme activity may be useful in detecting latent infection. Kit *et al.*



Text-figure 3.—KB cell DNA and Ad 2 DNA synthesis at different times after infection. At 12, 18, 24, 36, and 47 hours after infection, 300 ml amounts of cells were incubated with ^{3}H-thymidine for 3-hour intervals. Total cell DNA was isolated and centrifuged to equilibrium in CsCl density gradient with marker Ad 2 ^{32}P-DNA. Results are expressed as percentage of total radioactivity recovered from the gradient.

(*22*) have demonstrated increased enzyme activities in cells abortively infected with SV40. However, one difficulty with analysis of increased enzyme activities is the need for suitable baselines; *i.e.*, what is the enzyme level of a normal cell? The activities of enzymes of DNA synthesis are greatly affected by the biological state of the cells and their previous growth history.

RNA VIRUSES

Detection of the Parental Viral Genome

The detection of parental viral RNA in the infected cell is difficult. A possible method would be the hybridization of infected cell RNA with viral cRNA; *i.e.*, synthetic radioactive RNA complementary to viral RNA by RNA polymerase acting on a viral RNA template. However, this does not appear too promising, since RNA–RNA hybridization measurements are not as sensitive as DNA–RNA hybridization measurements and the latter are as yet not satisfactory for detecting parental viral DNA in infected cells.

Detection of Replicating Viral RNA or Viral Complementary RNA

If replication of viral RNA occurs in latent infection, then it may be possible to detect viral involvement by several methods:

1) *Actinomycin-resistant synthesis.*—Actinomycin D inhibits cell RNA synthesis but does not inhibit the synthesis of viral-specific RNA in cells infected by picornaviruses (*23*), arboviruses (*24*), reovirus (*25*), and myxovirus, such as influenza virus (*26*), and Newcastle disease virus (*27*). A general test for latent infection by these viruses would be to label cells with ^{3}H uridine in the presence of actinomycin D and analyze the labeled RNA by sucrose gradient centrifugation. The presence of radioactive RNA peaks absent from normal cells treated with actinomycin and characteristic of viral RNA would suggest the transcription of viral RNA.

2) *RNA–RNA homology.*—RNA complementary to viral RNA can be detected by annealing RNA isolated from cells with radioactive viral RNA. The formation of labeled RNA–RNA hybrids resistant to ribonuclease treatment would provide evidence for the synthesis of viral cRNA. Cells infected with Newcastle disease virus synthesize large quantities of viral cRNA (*27*, *28*), but other RNA virus-infected cell systems synthesize predominantly viral RNA (*29*). Viral RNA should be detectable by RNA–RNA hybrid formation with radioactive viral cRNA, though relatively little has been done with this type of analysis.

3) *Other procedures.*—High molecular weight RNA with a sedimentation coefficient of about 70 S is extracted from the virions of the murine and avian leukemia viruses (*30-32*). Uninfected cells do not possess stable RNA molecules of sedimentation coefficient higher than 30 S. Therefore it may be possible to detect latent infection with these viruses by examining cellular RNA for the presence of fast-sedimenting components. However, the usefulness of this test needs first to be established experimentally with cells infected with appropriate viruses.

Detection of Viral-Specific Proteins

It may be feasible to assay for the viral-specific RNA synthetase induced early after infection with picornaviruses (*33*) and arboviruses (*34*) using cell-free extracts of infected cells.

CONCLUSION

With the increased knowledge of the biosynthesis of animal viruses and the improved biochemical methodology now available, it is feasible to determine biochemically whether a cell is latently infected by those known viruses which can be conveniently grown and purified. The requirement for biochemical analyses in most cases is the availability of pure viral nucleic acids for use as reagents in homology tests. Biochemical methods of analysis have been successful in cells infected with, or transformed by, DNA viruses, but much less information is available on cells infected with RNA viruses.

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Detection of Viral Latency by Biochemical Methods—Discussion

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As IS eminently clear from the extensive data of Dr. Green and his collaborators, homology or hybridization studies can provide a great deal of information on the presence of viral genetic material in cells when one deals with a well-defined system. It must be emphasized, however, that the ability to detect a specific agent is dependent upon having available that agent in a highly purified form. Since it has been demonstrated by Thomas and co-workers and McCarthy and co-workers that only approximately 15 nucleotides need be in register to provide stable hybrids, the presence of minute amounts of contaminating DNA in the virus preparation may make interpretation of results impossible.

As Dr. Green already has indicated, Weil and co-workers and Winocour and co-workers have suggested that polyoma virions contain host-cell DNA. If this phenomenon should indeed appear to be a more general phenomenon, the interpretation of hybridization data obviously becomes an even more difficult problem.

Furthermore, the use of direct binding of either RNA or DNA to DNA generally provides qualitative rather than quantitative data on relationships between the agent and the cell or among specific agents. To achieve quantitative relationships (among the adenoviruses), such as has been demonstrated by Dr. Green, large numbers of determinations must be made in order to achieve significance. To those who are not familiar with Dr. Green's extensive publications, I should like to call your attention to the numbers of determinations he has made to provide the data on relatedness among adenoviruses. Twenty to thirty independent determinations are not unusual.

Although Beernbaum and Darnell have cast doubt on the validity of competition experiments with nucleic acids derived from mammalian cells, data obtained from properly conducted competition experiments can be extremely valuable. In addition, the criticisms which have been leveled at competition experiments do not relate to data obtained with small DNAs such as those of viruses or with the RNAs from these viruses. By the competition technique it is possible to evaluate two nucleic acid samples in a single experiment. Because of the obvious advantages of the identity of experimental conditions achieved via competition, these data take on additional significance.

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[1] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Biophysical methods for the isolation of viral nucleic acids whose expression has been masked have not been a fruitful area for exploitation. The obstacles are formidable when one considers the relative molecular weights of the DNAs of the host cell and the virus. One is searching for 1 part in 10 million at worst and 1 part in 10,000 at best.

There are techniques, however, which can be used to enhance one's ability to detect DNAs that differ from the bulk of the host-cell DNA. Fragmentation of host-cell DNA, followed by fractionation on OH apatite columns or by CsCl gradient centrifugations, allows one to select specific areas of base composition for examination. By examination of renaturation kinetics of these isolated fragments, it is possible to determine something of the size of the genome from which the fragment originated.

While the smaller papovaviruses with their circular DNAs have unique properties when they are examined in the purified isolated state, these properties apparently no longer exist when the virus genome becomes part of a transformed cell. The covalently linked circular DNAs of these molecules do not appear to be present. With the development of techniques for the separation of chromosomes, specific chromosomes may be implicated in the process of transformation. Efforts are being made to identify sites for preferential interaction of virus with host genes. These techniques may serve to enhance our ability to examine cellular DNAs for the presence of foreign agents.

Despite what has been said about the promise and the achievements of biochemical techniques in the detection of latent viruses, these techniques have to date provided little more than the direct demonstration or confirmation of what has been suggested by biological studies. Biochemical methods have sophistication but lack the flexibility of present biological techniques. Hopefully, with the application of some of the biological ingenuity, a more practical biochemical approach to the problem of latent viruses may be developed.

Detection of Persistent Infections Using Biophysical Methods[1]

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We are concerned with immunizing large numbers of people. The ultimate goal is a synthetic antigen or epitope. To reach that goal, the following steps may be listed:

1. Immunization with tissue virus mixture, as is presently done.
2. Purification of the virus.
3. Purification of the protein antigen or antigens.
4. Synthesis of the epitopic site.

We are at stage 2 and we must therefore consider the detection and elimination of nonviral particles from vaccines. Physical detection methods are less sensitive, usually by many orders of magnitude, than biological methods. The physical methods that may be used include fluorescent antibody staining and detection by sensitive optical means, including quantum counters, electron microscopy with or without previous fractionation, and particle distribution studies using centrifugation, adsorption methods, gel filtration, electrophoresis, or other separative methods.

Obviously, the prudent approach to the purification problem is to eliminate all possible adventitious infectious agents at the start, and then remove all remaining nonimmunogenic mass possible by physical and chemical methods. Pure virus particles can be isolated in the laboratory. The problem is the development of production-scale systems. This has been a concern of ours at Oak Ridge for a number of years.

I shall discuss first certain limitations of electron microscopy. We have developed counting methods applicable to pure suspensions having as little as 5×10^4 particles per ml. The usefulness of such methods falls off rapidly as the background contamination builds up. In previous studies we have considered the number of photomicrographs necessary to find virus particles in cells. For a cell 15 μ in diameter, with 250 A sections and 8 × 10 inch photographs at 20,000 ×, one particle would be seen in each picture, on an average, if there were 600 particles per cell and the tissue titer would be 3×10^{11} particles per g. If one particle were present per cell the titer would be 5×10^{8}, and an average of 600 photomicrographs would be needed to find one particle. This is not a sensitive method.

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[1] The Molecular Anatomy Program is sponsored by the National Cancer Institute, The National Institute of General Medical Sciences, The National Institute of Allergy and Infectious Diseases, and the U.S. Atomic Energy Commission.

[2] Operated for the U.S. Atomic Energy Commission by the Nuclear Division of Union Carbide Corporation.

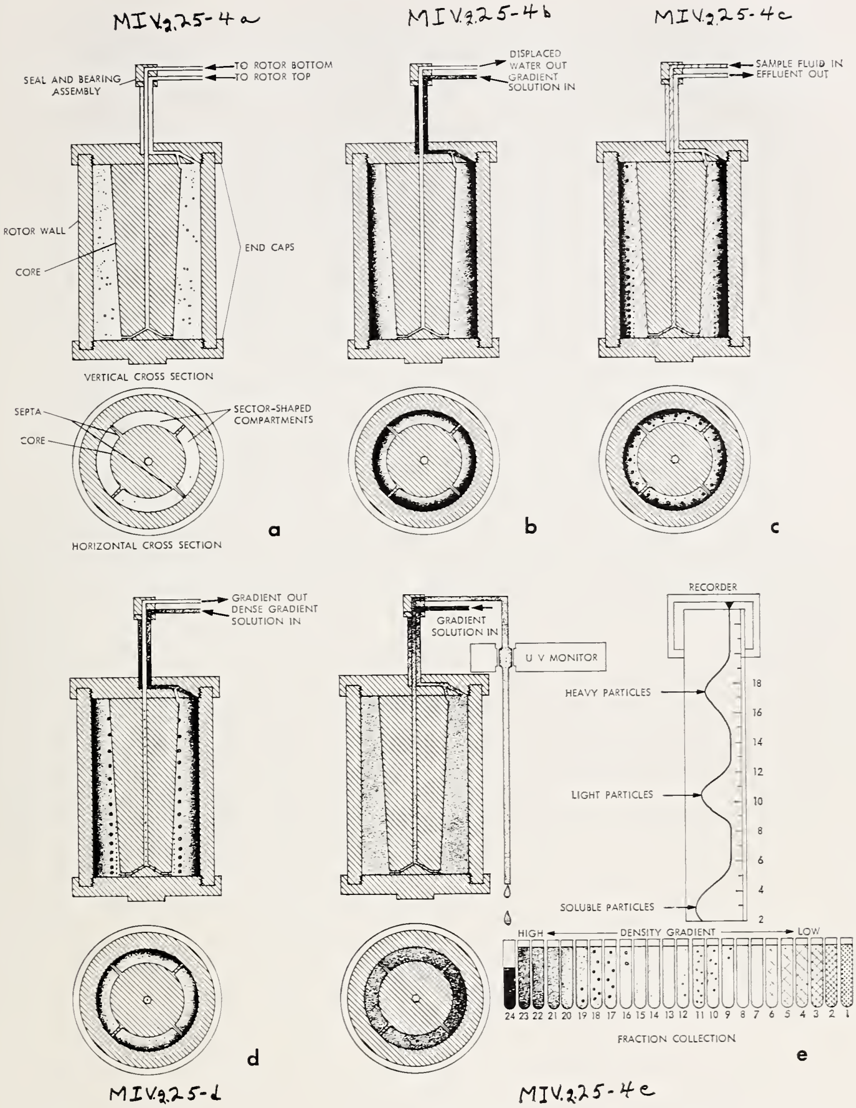
We have proposed instead, and spent many years developing, production scale methods for virus purification. By centrifugal methods the separations shown in figure 1 should be possible. An actual separation with a virus is shown in figure 2. By accident, the preparation contained two viruses which were separated as shown in figure 3. The separation of a virus from a liver homogenate is shown in figure 4. Virus particles of high purity were recovered in the small band at the tip of the arrow. Zonal rotor systems for rate and isopycnic banding purification of production of quantities of virus have been developed at Oak Ridge and are now becoming commercially available. However, the problem of concentrating the virus initially remained. New methods were required and a technique termed "continuous-sample-flow-with-banding" was developed and is illustrated in text-figure 1. Virus removal as a function of virus sedimentation coefficient and flow rate is shown in text-figure 2. A larger version of this system, the K-II centrifuge, is being developed for batch sizes of 100 liters and beyond. This system is shown in figure 5. Two of these units have been in use at the Eli Lilly plant at Indianapolis for some time.

Electron micrographs of batches of commercial vaccine have been made which show the presence of large quantities of nonviral contaminants. Studies with zonal centrifuge-purified influenza vaccines (fig. 6) show these contaminants to be almost totally absent. Elimination of known infectious agents does not by itself lead to purity. By using the centrifuges as described, a high degree of purity is routinely obtained with influenza virus.

Vaccine production is a problem in systems analysis and development, and the facilities of the 3 plant areas in Oak Ridge are available to us to attack these problems. Extensive research studies by 5 pharmaceutical firms, who are using Oak Ridge systems, are being carried out.

A word about subunit vaccines: If pure protein subunits can be isolated, does the cell or tissue of origin matter? The answer depends on our definition of purity and the demonstration that nucleic acid is absent. One approach is to add carrier impurities which can be detected and then to show they are removed. Work along this line might prudently be combined with attempts to produce virus-free calf serum and human plasma products.

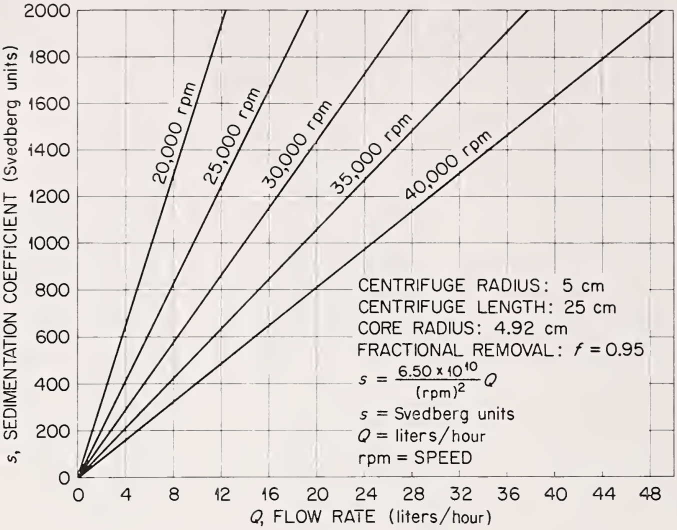
We must consider then the possible effects of taking a protein isolated from, for example, bovine sources, purifying it, and injecting it on a regular schedule into large numbers of children and adults for their whole


MIV2.25-4a
MIV2.25-4b
MIV2.25-4c
SEAL AND BEARING ASSEMBLY
TO ROTOR BOTTOM
TO ROTOR TOP
ROTOR WALL
CORE
END CAPS
VERTICAL CROSS SECTION
SEPTA
CORE
SECTOR-SHAPED COMPARTMENTS
HORIZONTAL CROSS SECTION
a
DISPLACED WATER OUT
GRADIENT SOLUTION IN
b
SAMPLE FLUID IN
EFFLUENT OUT
c
GRADIENT OUT
DENSE GRADIENT SOLUTION IN
d
GRADIENT SOLUTION IN
U V MONITOR
RECORDER
HEAVY PARTICLES
LIGHT PARTICLES
SOLUBLE PARTICLES
18
16
14
12
10
8
6
4
2
HIGH
DENSITY GRADIENT
LOW
24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1
FRACTION COLLECTION
e
MIV2.25-4d
MIV2.25-4e

TEXT-FIGURE 1.—Operational sequence of continuous-sample-flow-with-banding centrifuge. (From Anderson, N. G., Barringer, H. P., Amburgey, J. W., Jr., Cline, G. B., Nunley, C. E., and Berman, A. S.: Nat Cancer Inst Monogr 21: 214–215, 1966.)

lifetime. What requirements on virus and tissue nucleic acid should be set? (Parenthetically, one may ask, "What is the virus and nucleic acid content of insulin, a widely used tissue product?")

A consideration of all experience with biological products which are rigorously purified suggests that the cell of origin may be unimportant for subunit vaccines properly purified.



TEXT-FIGURE 2.—Theoretical relationship between sedimentation coefficients and flow rates at various speeds for 95% virus removal by using the B–V rotor. [From A. S. Berman, Nat Cencer Inst Monogr 21: 55, 1966.]

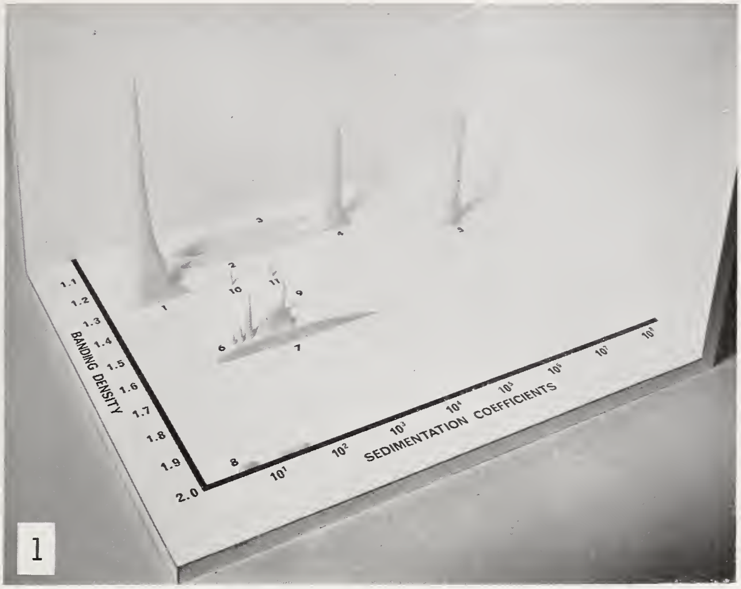


FIGURE 1.—Diagrammatic presentation of distribution of subcellular components and three viruses as a function of the sedimentation rate (in Svedberg units) and banding density (g/cc). The fractions shown include: 1—soluble proteins; 2—membranes with ribosomes; 3—smooth membranes; 4—mitochondria; 5—nuclei; 6—ribosomal subunits, ribosomes and polysomes; 7—glycogen; 8—RNA; 9—poliovirus; 10—T3 bacteriophage; and 11—adenovirus type 2. Banding densities for most particles are those for cecium chloride. The viruses shown fall in a relatively clear space which has been termed the "virus window." (From Anderson, N. G., *Science* 154: 104, 1966.)

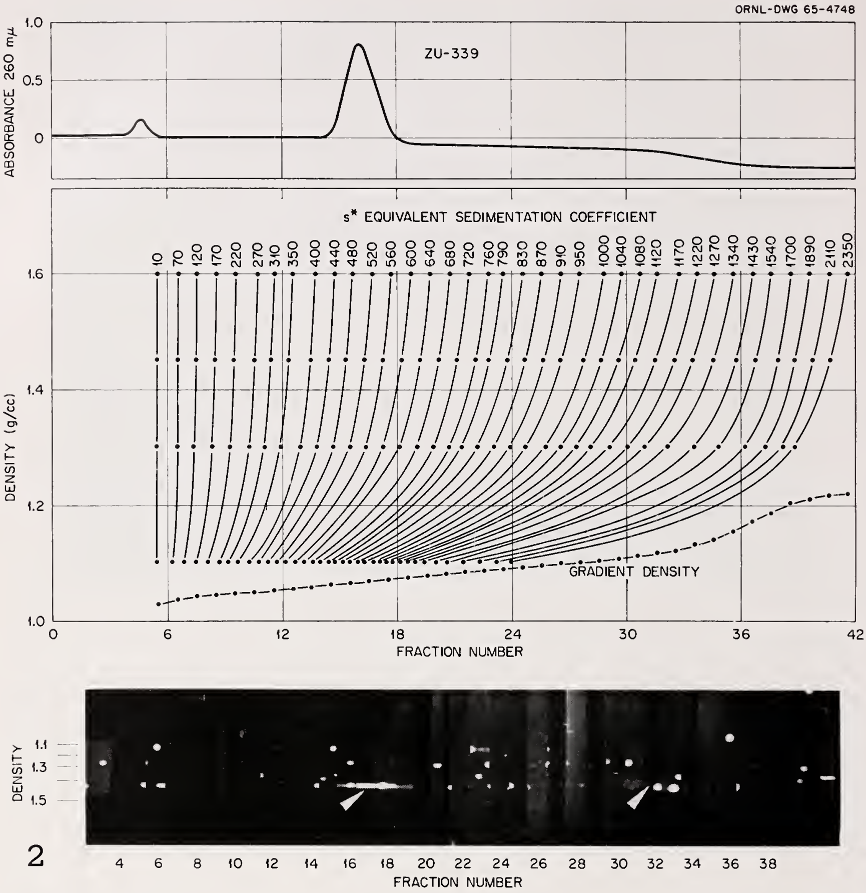


FIGURE 2.—Combined rate-zonal and isopycnic-zonal separation of T3 bacteriophage accidentally contaminated with T2 bacteriophage. The position of the two virus bands are indicated by *arrows* in the lower pycnogram. (From Anderson, N. G., Harris, W. W., Barber, A. A., Rankin, C. T., Jr., and Candler, E. L.: Nat Cancer Inst Monogr 21: 277, 1966.)

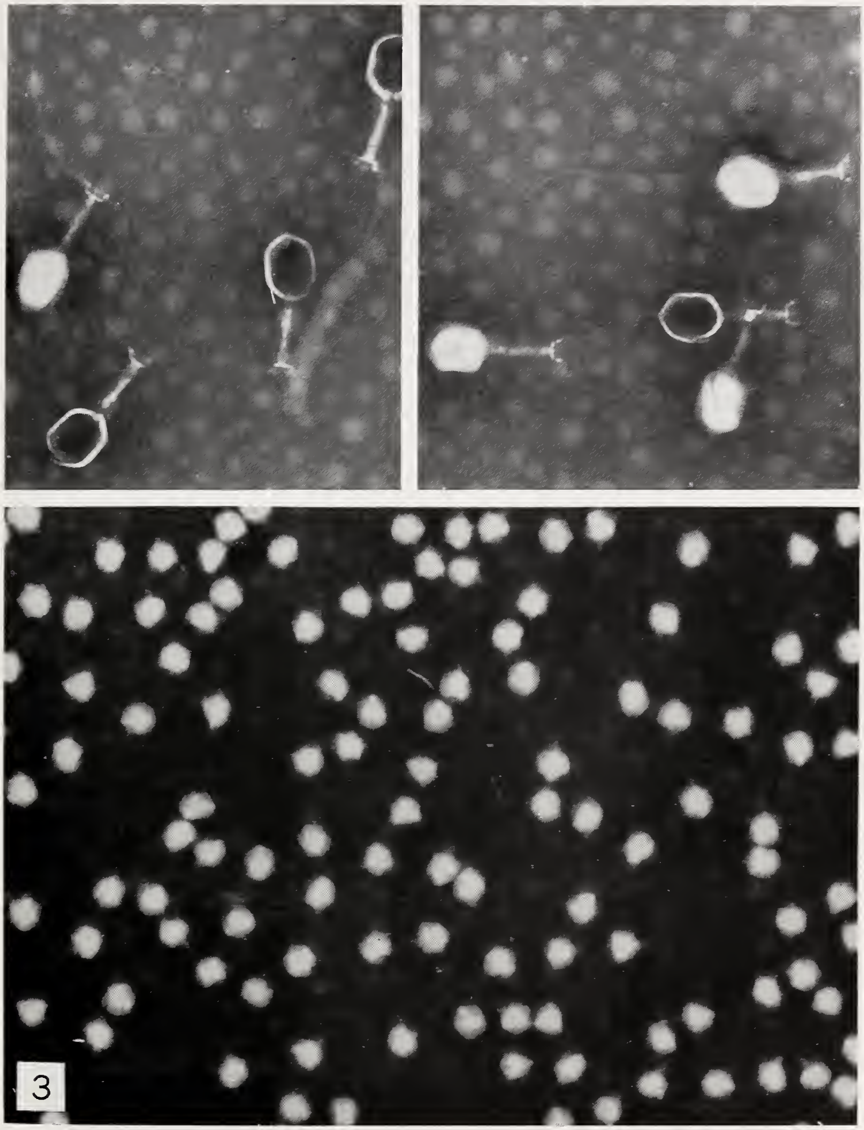


FIGURE 3.—T2 and T3 bacteriophage recovered from bands indicated in figure 2. *Lower* micrograph shows T3 particles from fraction 17; *upper* two micrographs show T2 particles recovered from fraction 32. × 80,000. (From Anderson, N. G., Harris, W. W., Barber, A. A., Rankin, C. T., Jr., and Candler, E. L.: Nat Cancer Inst Monogr 21: 278, 1966.)

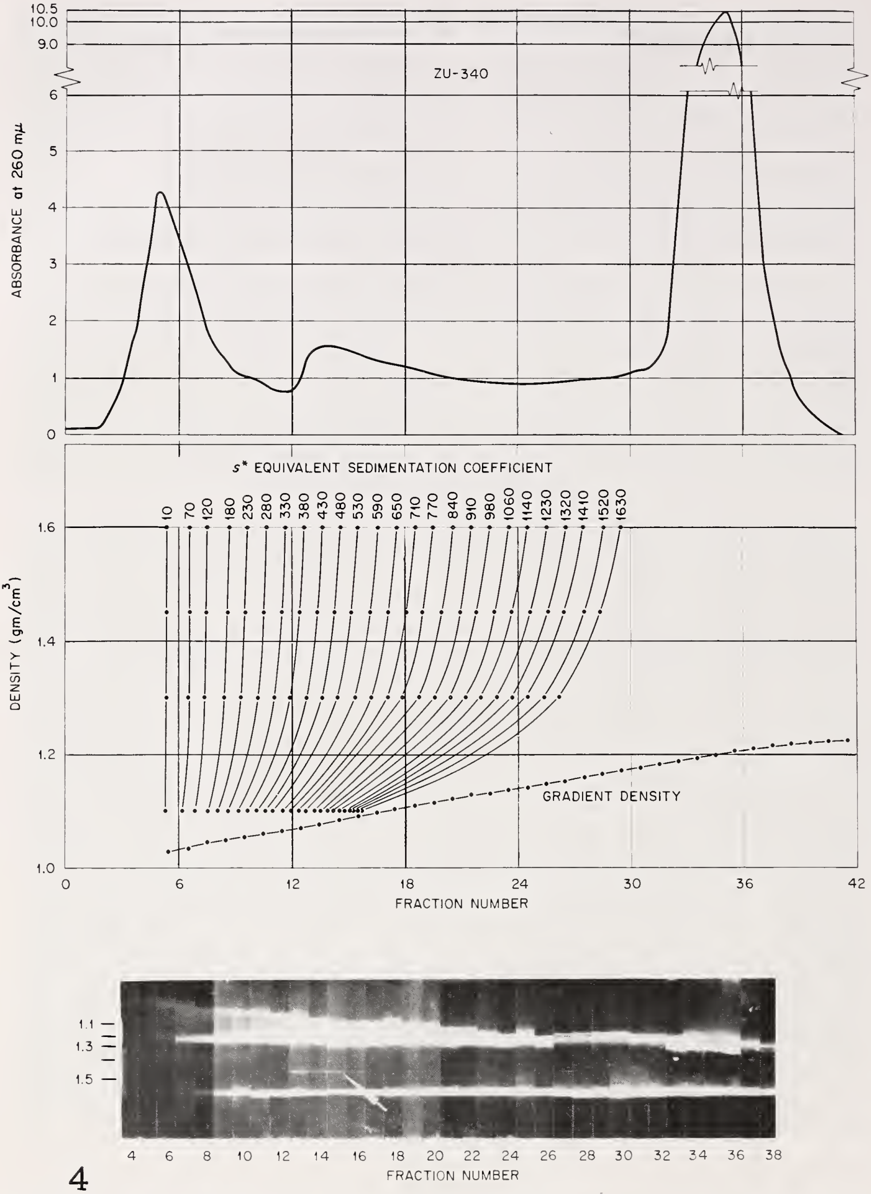


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Figure 4.—Recovery of added T3 phage from a rat liver brei by using the s-ρ technique. The particles from the band indicated in the *lower* pycnogram were found to be almost entirely T3 phage. (From Anderson, N. G., Harris, W. W., Barber, A. A., Rankin, C. T., Jr., and Candler, E. L.: Nat Cancer Inst Monogr 21: 279, 1966.)

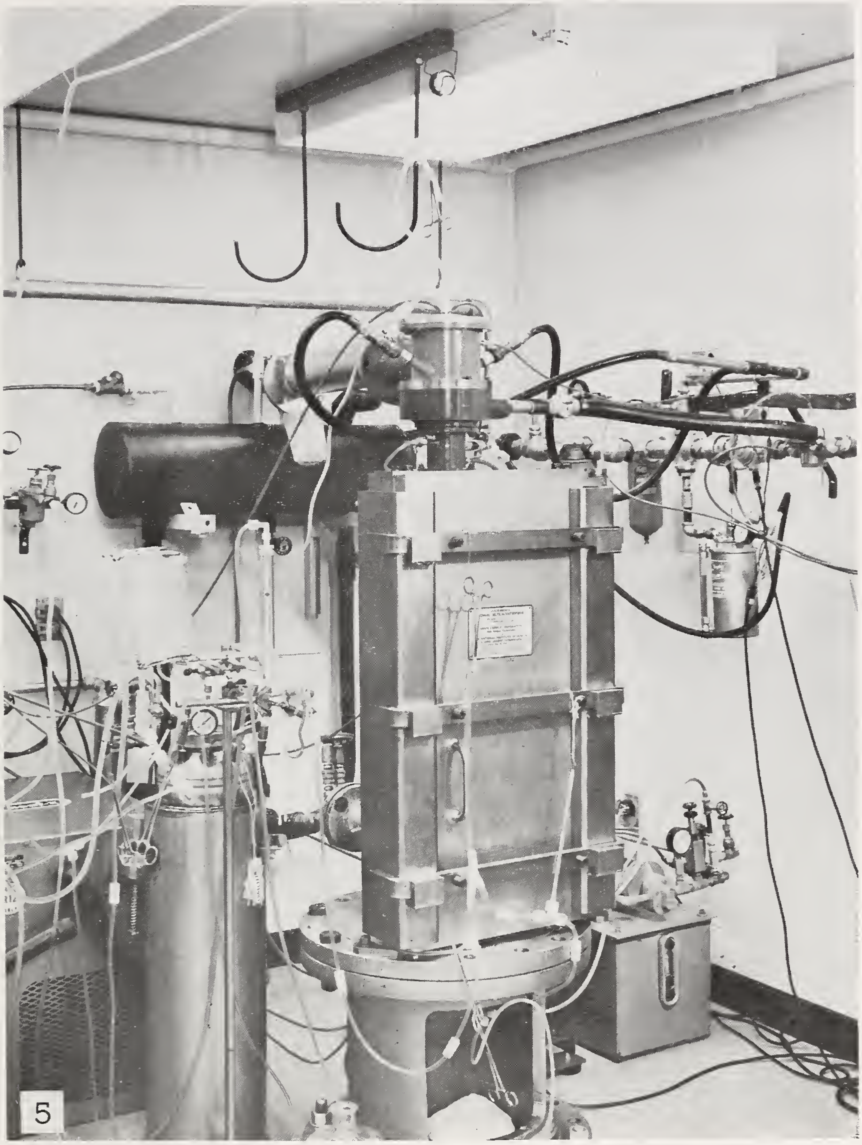

FIGURE 5.—K-II-B centrifuge developed at Oak Ridge under the joint National Institutes of Health-Atomic Energy Commission Program.

ANDERSON

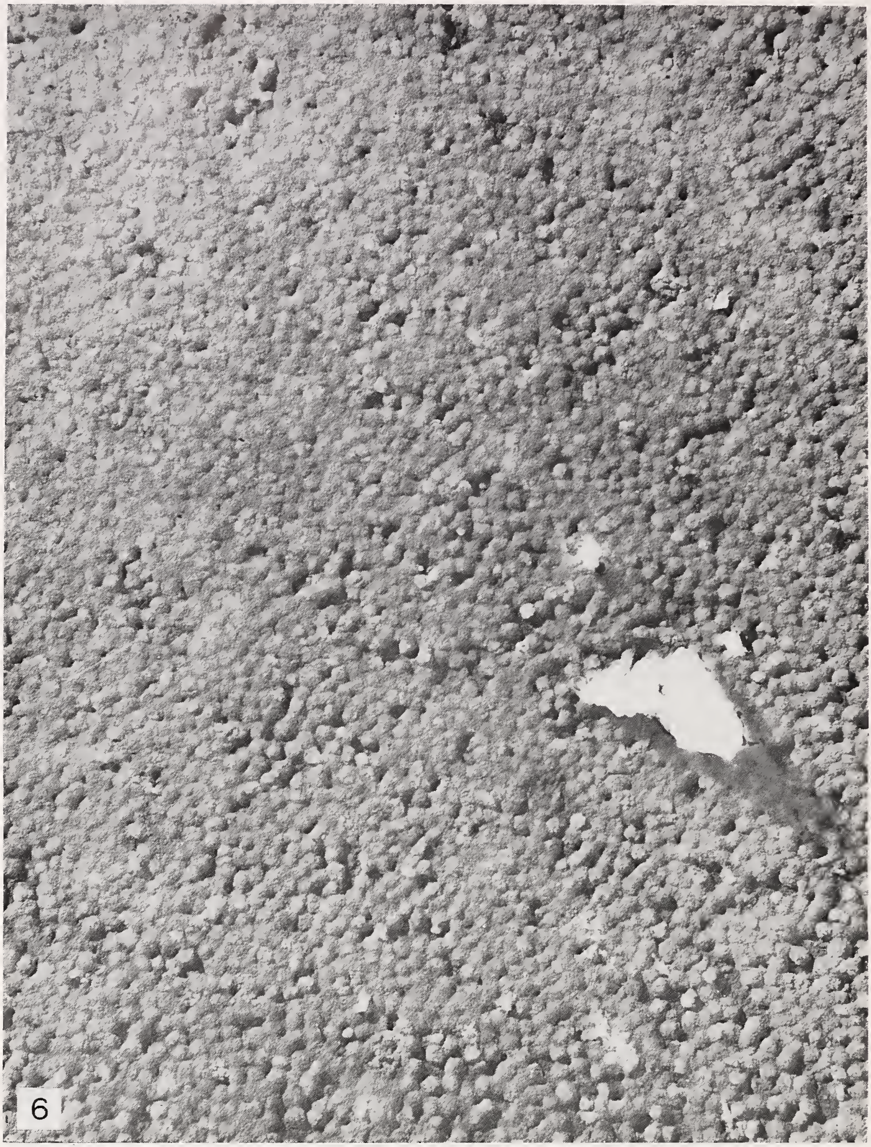

Figure 6.—Electron micrograph of monovalent vaccine (Md.B) purified by the combined process. × 37,200. [As described by Reimer, C. B., Baker, R. S., van Frank, R. M., Newlin, T. E., Cline, G. B., and Anderson, N. G.: J Virol 1(6): 1212, 1967.]

GENERAL DISCUSSION

Dr. K. O. Smith: Dr. Anderson, how do you achieve a 5×10^4 sensitivity for virus detection?

Dr. K. Habel: By electron microscopy, you mean?

Dr. Smith: Yes.

Dr. N. G. Anderson: We use very smooth, long, plastic tubes with an internal taper having the grid at the bottom. We centrifuge out of a large volume all the particles onto the grid. Thus by merely increasing the original volume and spending a lot of time looking, we can achieve this level.

Dr. Smith: But this assumes highly purified virus?

Dr. Anderson: As I said, this method works with highly purified virus, and the sensitivity or the applicability drops off rapidly as the trash background builds up.

Dr. Habel: But this is a total, 5×10^4 isn't it?

Dr. Anderson: Yes.

Dr. Habel: And the whole column?

Dr. Anderson: That is the concentration at the start per solution per ml.

Dr. J. V. Sorrentino (The National Drug Company) : Dr. Anderson, is the work you just reported on in purification all done in sucrose gradients?

Dr. Anderson: The continuous flow with banding is usually done in sucrose gradient, although we have worked with cesium chloride and sucrose-cesium chloride combinations.

Dr. Sorrentino: Is the rotor core anodized aluminum or some other material?

Dr. Anderson: We have used the aluminum rotors with cesium chloride for quite some time. The commercial manufacturers recommend that this not be done, probably because a number of users have employed acid cesium chloride and dissolved their rotors.

The newer systems that we are developing are made of titanium to obviate this difficulty. The B–XIV and B–XV rotors, for example, are titanium .The next generation of K–II rotors, which will be K–III and K–IV, will be titanium throughout. These can be steam-sterilized.

Satellite Viruses, Helper Viruses, and Synergistic Action[1]

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When two viruses infect the same cell, several types of interaction are possible. First, the double infection may have no effect on the replication of either virus; each virus can replicate as it would in a single infection. Second, interference may occur; that is, the previous infection of cells with one virus prevents the replication of the superinfecting virus. The third type of interaction, the one to be summarized in this report, involves enhancement of virus production in mixedly infected cells. This stimulation of virus synthesis includes (*a*) that provided by a companion virus to satellite viruses found only in the presence of the first virus, (*b*) that provided by one virus (helper) to another virus during joint infection, and (*c*) the reciprocal complementation (synergism) that enables two defective viruses to replicate under otherwise inhibitory conditions.

SATELLITE VIRUSES

Associated viruses are morphologically and serologically unrelated to the viruses with which they may be found. Sometimes they grow independently of the companion virus, *e.g.*, poliovirus and SV40 in monkey kidney cells. Of particular interest for this Conference are the satellite viruses of tobacco necrosis virus (*1, 2*) and of adenovirus (*3–7*). In both the plant and animal virus systems the smaller satellite virus can be separated from the larger virus by biophysical means such as centrifugation to

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[2] American Cancer Society Professor of Virology.

[3] Dr. Janet S. Butel and Miss Maryann Jerkofsky deserve the gratitude of the author for their help in summarizing the information cited in this report.

equilibrium in density gradients. However, neither of the satellite viruses can replicate alone. Satellite particles cannot be detected in tobacco plants inoculated with this virus alone, although the particles are readily detected if the tobacco necrosis virus is present. Similarly, replication of the adeno-associated satellite remains undetected unless a replicating adenovirus is present. Like the satellite of tobacco necrosis virus (*1*, *2*), the satellites of adenoviruses can be detected (fig. 1) by electron microscopy (*3*, *5*, *7*, *8*). It can also be detected by complement fixation (*3*, *7*, *8*) and by immunofluorescence techniques (*9–11*). Some types can also be detected by hemagglutination of erythrocytes (*12*). Smith (*13*) discusses in greater detail the sensitivity of various methods used to detect the defective satellite virus associated with adenoviruses.

The requirement for the larger companion virus is specific; closely related plant viruses will not allow the satellite of tobacco necrosis virus to replicate (*1*). Any of a number of adenoviruses, but *only* adenoviruses will permit the replication of its satellite. The replication of the associated satellite virus interferes with the replication of the companion virus, however. Higher titers of tobacco necrosis virus are obtained from plants inoculated with this virus alone, when compared with titers from plants that have also been inoculated with the satellite (*2*). Similarly, there is a 90% reduction in the titer of adenovirus in cells that also contain satellite particles (*7*, *8*, *14*).

The exact mechanism of interaction between the associated viruses is not known. Analysis of the RNA of the plant satellite shows that it can code for a maximum of 100 amino acids (*15*). This corresponds to the number of amino acids contained in its coat protein. Obviously, however, these 100 amino acids are insufficient for the replication of the virus; at least one function must be provided by the tobacco necrosis virus. The mechanism of interaction between adenovirus and its satellite has not been clearly defined. Quantitative studies of the growth kinetics of the satellite have shown that its latent period can be shortened to 4–6 hours if the cells are preinfected 12 hours earlier with adenovirus (*8–10*). If the synthesis of the late viral capsid antigens is prevented in doubly infected cells by treatment with flourodeoxyuridine (FUDR) and then reversed, the satellite capsid antigen can be detected before that of the adenovirus capsid antigen (*10*). Therefore, the functional interaction of these two viruses does not appear to involve a terminal step in the synthesis of the adenovirus capsid antigen. However, adenovirus DNA synthesis alone will not permit the replication of its satellite virus; no replication occurs in simian cells abortively infected with a human adenovirus (*10*), a system in which large amounts of virus-specific DNA are formed (*16*). Therefore, the critical event in the interaction between adenovirus and its satellite appears to occur after adenovirus DNA synthesis, but before the terminal steps of adenovirus capsid antigen formation.

HELPER VIRUSES

When the multiplication of one virus allows another virus to complete its replication, the former is said to be a "helper" virus. The conditions under which such assistance is provided must be specified; a helper virus may be essential in one cell type while the enhanced virus is often capable of independent replication in another cell system. The helper virus is usually not found with the second virus under natural conditions, but is added in the laboratory. However, the Rous-associated virus (RAV)-Rous sarcoma helper system is an exception to this generalization.

Newcastle disease virus replicates only slightly in calf kidney cells because an interferon-like substance present in the inoculum produces autointerference (*17*). Coinfection of the calf kidney cells with parainfluenza virus type 3 enhances the replication of Newcastle disease virus. In this case, the exact mechanism of action is known. The parainfluenza virus type 3 both inhibits the antiviral activity of the interferon-like substance in the inoculum and suppresses the formation of interferon by the infected cells. The same mechanism of interaction may occur in swine testicular cells; Newcastle disease virus cannot replicate in these cells unless they are jointly infected with hog cholera virus (*18–20*).

Enders, Holloway, and Grogan (*21*) have recently reported that Sendai virus which has been irradiated with ultraviolet light will allow poliovirus to replicate in normally resistant chicken embryo and hamster embryo cells. Apparently, polio virions are mechanically incorporated into the cytoplasm of the otherwise resistant cells during fusion of the cells induced by Sendai virus.

Another example of a helper virus involves the "defective" Rous sarcoma virus (RSV). The Bryan high titer strain of RSV is defective in that greater than 80% of the transformed foci produced by this virus in cultures of chicken cells fail to produce virus, even when treated with X irradiation or sonic vibration (*22*). These cells are called nonproducer cells. The transformed foci that do release RSV also release another leukosis virus, RAV. RAV is an avian leukosis virus that was found in the Bryan high titer stock of RSV (*23*). Superinfection of nonproducer cells with RAV (*22*) or with other members of the avian leukosis group of viruses (*23–25*) will cause the nonproducer cells to release virus which can cause typical Rous sarcomas in chickens. The virus released by these procedures has the growth characteristics (*26*), host range (*27*), and antigenic characteristics (*28*) of the added helper virus.

RSV alone can produce and maintain the malignant transformation of normal chicken embryo cells to sarcoma cells (*22*, *28*). Therefore, it appeared that the production of mature virus was not needed for transformation. Viral coat protein of RSV has not been detected by immunological techniques in the nonproducer cells (*28*); such antigens are readily detected if RAV is present. All these findings seemed to indicate that RAV was needed to form the viral capsid which the defective Bryan strain of

Rous sarcoma apparently could not produce. Recently, however, virus-like particles have been detected in nonproducer cells (*29*) and both noninfectious particles with chemical and physical characteristics similar to those of a mixture of RSV and RAV (*30*) and a small number of infectious RSV particles (*31*) have been isolated from nonproducer cells. The use of a different indicator cell system (*32*) has revealed that the particles released by nonproducer cells are *not* defective and that the previous observations are the result of phenotypic mixing of RSV and various avian viruses. These findings are described in greater detail by Vogt (*32*).

The coat protein of RSV appears to limit the expression of its oncogenic potential. The Schmidt-Ruppin strain is oncogenic in hamsters but the Bryan strain is not. If the Schmidt-Ruppin genome is coated with the capsid of RAV–1, the strain loses its ability to produce tumors in hamsters. If the Bryan strain genome is carried in capsids of the recently isolated helper virus, RAV–50 (*33*), tumors are then produced in hamsters. No tumors are produced when the Bryan strain is coated with RAV–1 or RAV–2 protein coats. Therefore, it appears that the coat protein of the virus may limit viral expression, perhaps by controlling viral adsorption, penetration, or uncoating.

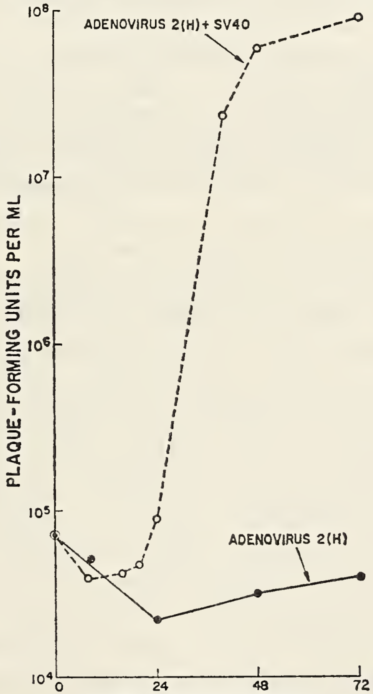
A system similar to RSV has recently been described with Moloney sarcoma virus (MSV). Suspensions of MSV consist of two types of particles—focus-forming particles and non-focus-forming particles (*34*). Both particles appear necessary for focus formation in tissue culture, although confirmation of the original report is still lacking. However, if an excess amount of Moloney leukemia virus (MLV) is added, only one particle is required for focus formation. Virus can be recovered from tumor cells by the addition of other murine leukemia viruses, *i.e.*, Rauscher, Friend, Moloney, to mixed cultures of MSV-induced hamster tumor cells and normal mouse embryo fibroblasts (*35*), although no virus can be recovered directly from the tumor cells in the presence or absence of the helper leukemia virus. The recovered sarcoma virus has the antigenic characteristics of the added helper virus. Therefore, it seems that the helper virus may function in providing the capsid protein.

Human adenoviruses undergo an abortive infection in simian cells. They adsorb, penetrate, and induce the formation of the adenovirus-specific tumor (T) antigen (*36*, *37*) and virus-specific DNA (*16*). However, no viral capsid antigen (table 1) can be detected (*36*, *37*). If the simian papovavirus SV40 is added to the monkey cells, the adenovirus capsid antigen can then be detected and infectious adenovirus (text-fig. 1) is produced (*38*–*45*). Simian adenoviruses SV15 and SA–7 also allow the human adenovirus to undergo a complete replicative cycle in the simian cells (*46*) but herpes simplex and other viruses have failed to act as helpers (*36*).

The exact mechanism of the interaction of the human adenovirus with the simian viruses is not known. However, further analysis of the abortive replication of the human adenoviruses has shown that the degree of defectiveness varies with the adenovirus (*47*). With adenovirus type 2, there is little or no increase in the titer of infectious virus above the eclipse level, whereas with adenovirus type 7 a small amount of infectious virus is produced, the amount depending on the level of the inoculum. It appears likely that a step in the transcription or translation of late events in the cycle of the virus is not performed efficiently in the simian cells in the absence of helper viruses.

TABLE 1.—Effect of SV40 on replicative cycle of human adenoviruses in simian cells

Property	Absence of SV40	Presence of SV40
Synthesis of adenovirus tumor antigen	+	+
Synthesis of adenovirus DNA	+	+
Synthesis of adenovirus capsid protein	0	+
Synthesis of adenovirus particles	0	+
Synthesis of infectious adenovirus	0	+



TEXT-FIGURE 1.—Replication of human (H) adenovirus type 2 in green monkey kidney cells in the absence and presence of simian papovavirus SV40. There is a marked increase in plaque-forming units of the human adenovirus (as measured by assay in human embryonic kidney cells) only when the simian cells are coinfected with SV40.

RECIPROCAL COMPLEMENTATION (SYNERGISM)

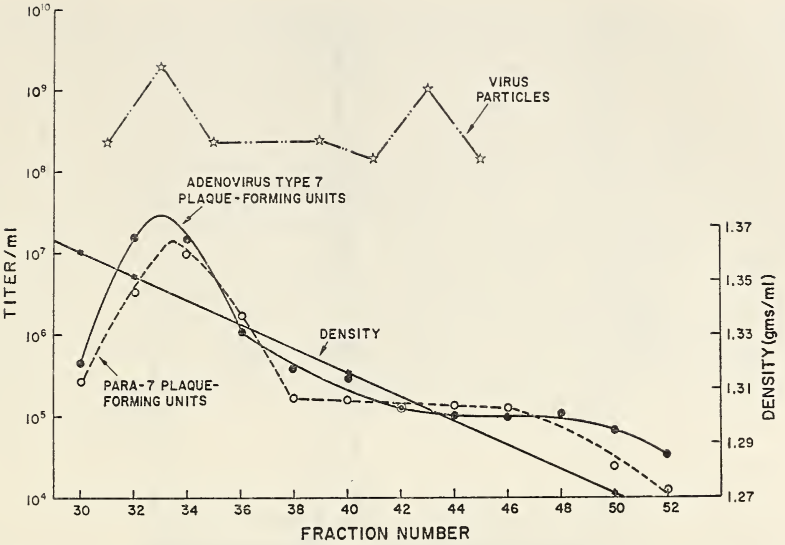
The final type of viral interaction to be discussed is the reciprocal complementation of defective viruses: Neither virus can replicate alone but both viruses replicate in doubly infected cells. The best characterized system involves the human adenoviruses and defective SV40 genetic material (PARA). This defective virus was detected in stocks of human adenovirus 7 that had been "adapted" to replicate in simian cells (*48–50*). The virus population can induce the synthesis of the T, but not the capsid, antigen of SV40. Since all detectable SV40-specific biological activity is neutralized by antisera to the adenovirus, it is believed that the SV40 genetic material is encased in adenovirus protein coats.

The two defective components (the adenovirus and the defective SV40) are in separate physical particles (*51–53*). Plaque formation in simian cells requires the presence of both particles; the infectivity dilution curves follow two-hit kinetics. If excess human adenovirus is added to the mixture of adenovirus and PARA, the infectivity dilution curve in simian cells converts to that of one-hit kinetics. In the presence of excess helper adenovirus, therefore, the defective PARA particle can be assayed quantitatively in monkey kidney cells. In contrast, only one particle is required for plaque formation in human cells. Virus progeny from plaques picked from human cells can neither induce the SV40 T antigen nor replicate in simian cells. Therefore, the defective SV40 genome is lost through plaque purification in human cells and the purified virus derived from human cells has all the properties of a human adenovirus.

The addition of an excess of another serologic type of human adenovirus during replication in simian cells allows the PARA particle to be transferred to a heterologous adenovirus population (*54*, *55*). Since the PARA particle is then carried in the capsid of an antigenically distinct adenovirus, this process has been called transcapsidation (*55*).

Although the adenovirus and PARA genomes are apparently carried in separate virus particles, various techniques to date have failed to achieve a physical separation (text-fig. 2) of the two components (*45*, *56*). Biophysical techniques have similarly failed to separate the adenovirus DNA from the SV40 DNA in nucleic acid preparations extracted from PARA-adenovirus 7 (*57*, *58*) or from PARA-adenovirus 2 (text-fig. 3).

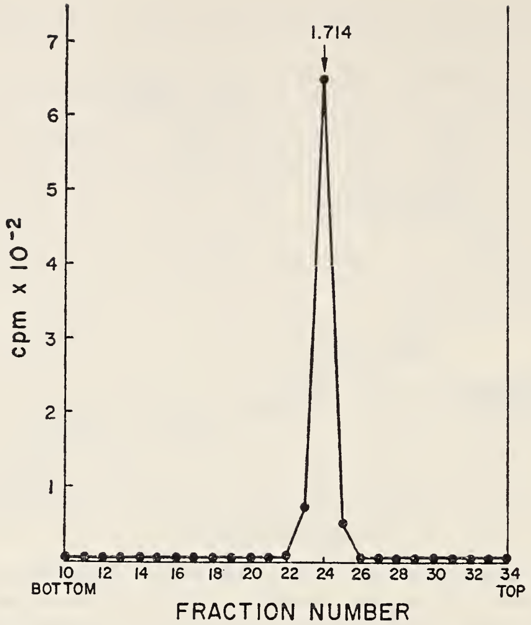
Replication of the PARA particle cannot be detected in the absence of adenovirus, but in doubly infected cells the two components replicate in parallel (*39*). The growth characteristics resemble those obtained with a mixed infection of human adenovirus and complete SV40 in monkey cells. It appears that the adenovirus provides coat protein for the PARA genome. The defective PARA genome, in return, fulfills the same role as complete SV40. However, the nature of this SV40 function has yet to be elucidated. Some of the pertinent properties of SV40, adenoviruses, and PARA-adenoviruses are summarized in table 2.



TEXT-FIGURE 2.—Characterization of PARA (the particle carrying the defective SV40 genome) and the accompanying adenovirus type 7 by density gradient centrifugation in cesium chloride. Both particles have a density of approximately 1.34 g/ml, as measured by titration of infectivity. *Upper curve* depicts particle counts which peak at densities of 1.34 g/ml (full adenovirions) and 1.30 g/ml (empty adenovirions). From Butel and Rapp (*56*).

Early studies had shown that the original PARA-adenovirus 7 population was oncogenic in newborn hamsters (*48*) and that this oncogenic potential was transferred with the PARA particle to the previously nononcogenic human adenovirus type 2 (*59*, *60*). However, all human adenovirus populations that acquire the PARA particle are not able to induce tumors in hamsters (*61*); some transcapsidant populations are rendered highly oncogenic (types 1, 2, 5, and 6) upon the addition of the PARA particle, whereas others are not (types 3, 14, 16, and 21). One possible explanation for this discrepancy is that the difference in the adenovirus protein coat prevents adsorption and penetration of certain PARA-adenovirus populations, thereby limiting the expression of their oncogenic potential, an explanation already noted for tumor production by RSV (*33*). However, all PARA-adenovirus populations are able to induce SV40 transplantation immunity; that is, all populations, whether oncogenic or not, will protect hamsters from challenge with SV40-transformed hamster cells. This would indicate that adsorption and penetration of the PARA-adenovirus occurred in each case (*59*, *61*). Tumors induced by the oncogenic PARA-adenovirus populations were found to contain antigenic elements of both particles (*59–62*). Therefore, some interaction between the information in the particles in the PARA-adenovirus populations may be required to initiate or maintain transformation.

VIRUS VACCINE PRODUCTION



TEXT-FIGURE 3.—The density of DNA extracted from PARA-adenovirus type 2. Approximately 10 μg of purified DNA extracted from purified PARA-adenovirus type 2 labeled with ^{3}H-thymidine was centrifuged to equilibrium in cesium chloride. Fractions were collected from the bottom of the tube and the radioactivity and density of each fraction were determined. Maximum counts were obtained at a density of 1.714 a density similar to that of purified adenovirus type 2 DNA. *Note* that a second species of DNA (for PARA) was not detected by the methods employed. From Rapp and Khare (*58*).

TABLE 2.—Summary of some known properties of SV40, adenovirus, PARA-adenovirus, and monkey cell-adapting component (MAC)-adenovirus populations

Property	SV40	Adeno	PARA-adeno	MAC-adeno
SV40:				
mRNA	+*	0†	+	?
DNA	+	0	+	0
Tumor antigen	+	0	+	0
Viral antigen	+	0	0	0
Transplantation antigen	+	0	+	0
Adenovirus:				
mRNA	0	+	+	+
DNA	0	+	+	+
Tumor antigen	0	+	+	+
Viral antigen	0	+	+	+
Transplantation antigen	0	+	+	+

*+ = possesses property.
†0 = does not possess property.

One other system of reciprocal complementation has been well studied. It involves the human adenoviruses and another defective particle (MAC, for monkey cell-adapting component) that allows human adenoviruses to replicate in simian cells (*63*, *64*). This population behaves very much like the PARA-adenovirus populations. The origin of the defective MAC particle is not known. It does not induce any of the SV40 antigens, its DNA does not hybridize with SV40-specific mRNA (*65*), and it has not yet been linked to any known virus. MAC is important in that it indicates that the association of defective genomes with human adenoviruses is probably not limited to SV40. It also illustrates that a screening test based on the detection of known early virus-induced antigens is not sufficient to detect all contaminating defective genomes. The properties of MAC are summarized and contrasted with those of PARA in table 2 and the following list.

Similar Properties of PARA and MAC

1. Enhance replication of human adenoviruses in simian cells.
2. Dependent on helper adenovirus for replication.
3. Encased in adenovirus capsids.
4. Can be transcapsidated to other adenoviruses.
5. Lost on plaque purification in human cells.
6. Buoyant density of 1.34 g/ml in CsCl.
7. More sensitive to UV than SV40 or adenovirus.

DISCUSSION

Although other examples of the phenomena discussed in this report might have been cited, those most intensively studied have been used as models. The enhancement and helper activity supplied by some viruses to other viruses has obvious importance as regards production of virus stocks for vaccines. Adaptation of viruses to previously resistant cells, once thought to represent mutation and selection of the virus or cell, is obviously often mediated by the acquisition of foreign determinants. The selection that follows results in a stable virus population with an increased spectrum of activity, sometimes accompanied by the addition of tumorigenic potential. The origin of certain associated viruses like SV40 and paramyxovirus in poliovirus stocks grown in monkey kidney cells is clear. However, the origin and role of other associated viruses remain unclear, and their unwelcome presence in many adenovirus populations forces a new look at seed stocks and at the progeny ultimately to be used in vaccine production. The methodology already developed to detect associated viruses, either as nonrelated contaminants, as satellites, or as foreign determinants, must be improved and expanded as virus populations are certified for use in the production of vaccines for human subjects.

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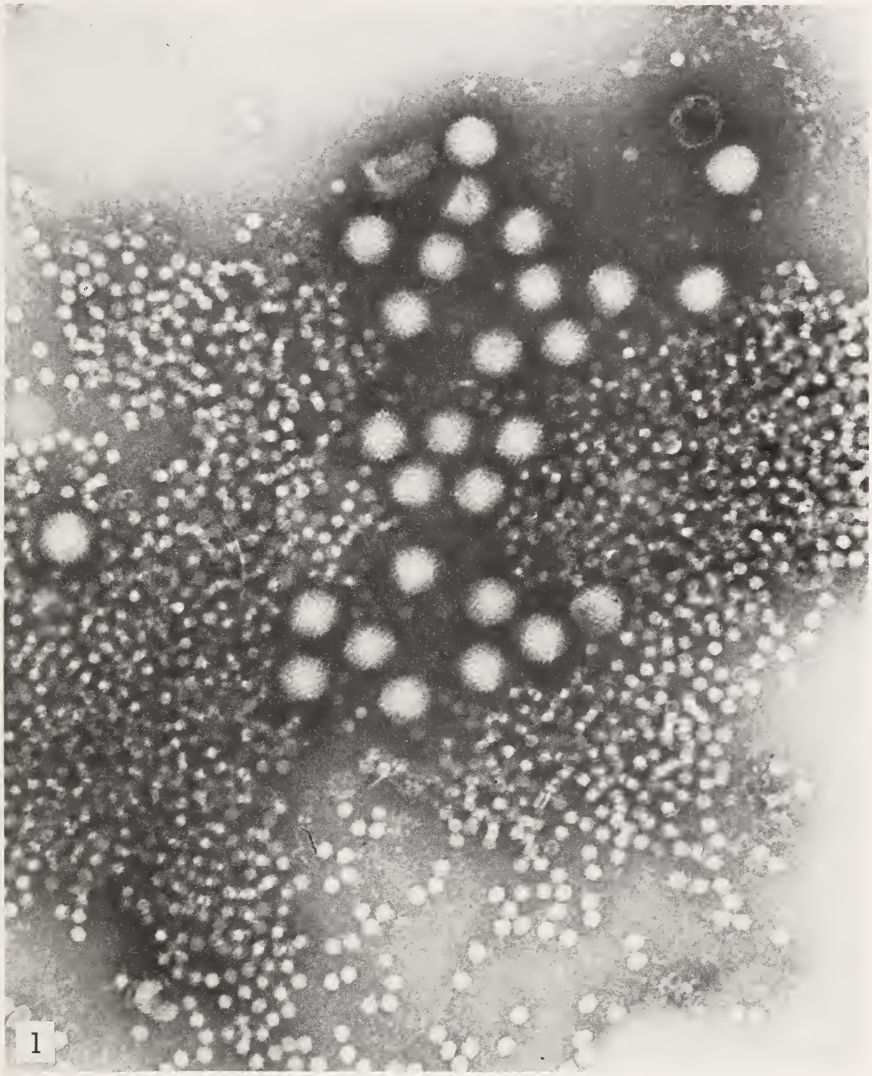

FIGURE 1.—Adenoviruses and smaller satellite particles. × 150,000

Electron micrograph supplied by Dr. Wade Parks and Dr. Joseph L. Melnick.

Cooperative and Antagonistic Interactions Among RNA Tumor Viruses[1]

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Recent studies on DNA as well as RNA animal viruses have uncovered a variety of situations in which a hitherto unknown virus was found to occur frequently in the company of a well-known and well-studied virus (*1–3*). The new agents are not more inert contaminants, but enter into various forms of cooperative or antagonistic interaction with the principal virus in whose company they are found. The cooperative interactions are not synergistic in the sense of a mutual dependence. One of the partners is capable of multiplying independently, and thus assists the replication of the other in the same cell (*2*, *4*). This cooperation accounts for the relative stability of the association between the two viruses. Under different conditions the same two viruses may interact antagonistically, resulting in interference with the replication of one of the partners. Reciprocal as well as nonreciprocal interference has been observed (*1*, *5–7*).

In the case of adenoviruses and adenosatellite viruses, no close relationship between the partners has been detected (*8*). On the other hand, RNA tumor viruses and their associated agents are closely related to each other structurally as well as antigenically (*1*, *9*). In order to differentiate between these two fundamentally dissimilar situations, it may be advisable to reserve the term "associated viruses" for those systems in which the partners are closely related and to refer to the unrelated companion viruses as "satellite viruses," a usage which has already been widely adopted.

The subject of this brief survey is the associated viral agents found in preparations of RNA tumor viruses. Besides their close relationship to the principal virus, these agents invariably show a significant, but often low, leukemogenic potential as outlined below.

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[1] Supported by Public Health Service research grant CA 10569 from the National Cancer Institute and by grant E 302 from the American Cancer Society, Inc.

Characteristics of Associated Viruses Found in the Company of RNA Tumor Viruses

1) Associated viruses are structurally and antigenically closely related to the principal virus in whose company they are found.
2) Associated viruses are not inert contaminants, but enter into cooperative and antagonistic interactions with the principal virus.
3) Associated viruses may replicate independently of the principal virus and show significant leukemogenic activity in susceptible host animals.

In fact, all the naturally occurring RNA leukemia viruses may become associated with RNA sarcoma viruses (*4, 10*). There are further associations between the leukemia viruses themselves involving agents of different leukemogenic potential. Stocks of avian myeloblastosis virus contain an apparently poorly leukemogenic agent capable of interfering with myeloblastic transformation of hematopoietic tissue *in vitro* (*11*). This virus has been isolated and studied (*12*). Other examples for this type of association are found among murine leukemia viruses (*13*). The models for associated agents of RNA tumor viruses are the various types of Rous-associated virus (RAV). These are avian leukosis-like agents that have been studied extensively with respect to their interactions with the Bryan high-titer strain of Rous sarcoma virus (RSV). The following discussion will be based on the RSV–RAV system. There is evidence that the basic findings are also applicable to the mouse sarcoma and leukemia viruses (*10*).

COOPERATIVE INTERACTIONS OF AVIAN SARCOMA AND ASSOCIATED VIRUSES

When a single particle of RSV infects a chicken cell, the virus induces neoplastic transformation but appears to lack the ability to direct the complete synthesis of infectious progeny virus. The cell-free medium of such Rous sarcoma cells shows no focus-forming activity if plated on cells susceptible to the parent virus (*4*). Recent work suggests, however, that some progeny virus is made by these Rous sarcoma cells, but that this virus differs from the parental virus in some important biological properties to be discussed below (*14, 15*). The old term "nonproducing cells" was therefore replaced by "L–R cells," an abbreviation for leukosis-free Rous sarcoma cells.[2]

Addition of an RAV to L–R cells results in an apparent enhancement of RSV release (*4*). This "helper" action of RAV controls the rate of RSV synthesis and the final RSV titer (*16*). However, the helper-independent production of RSV precursors may in some cases become the rate-limiting step in the maturation of RSV (*15*).

In addition to increasing the quantity of the released RSV, RAV also has a profound effect on the quality of the RSV produced. This effect reflects the mode of helper virus action, which is to displace the RSV envelope antigen with that of RAV. As a consequence of this interaction, the helper virus controls the envelope properties of RSV. These include antigenicity, host range, and sensitivity to viral interference. The subject of helper-dependent properties of RSV has been dealt with exhaustively in other publications (*17*, *18*), and is therefore merely summarized below.

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[2] H. Hanafusa (1967), personal communication.

Effect of RAV on Envelope Properties of RSV

Property	*References*
Control of type-specific antigen	(*9, 19, 20*)
Control of host range	(*21–24*)
Control of sensitivity to interference	(*7, 21, 22*)

Some strains of RSV replicate well in the absence of a helper virus and are considered nondefective in contrast to the Bryan high-titer strain. Examples are the Prague and the Schmidt-Ruppin strains of RSV (*25–28*). Nevertheless such helper-independent strains may carry associated viruses, as was demonstrated by the isolation of RAV–50 from the Schmidt-Ruppin strain of RSV (*24*). The defectiveness of the Bryan high-titer strain, until recently believed to be absolute, appears now to be the only relative (*14*, *29*, *30*). Most of the L–R cultures, initiated with RSV, release a virus capable of transforming certain avian cells (*15*, *31*, *32*). This virus differs from the helper-controlled parental RSV in its restricted host range, which explains why this agent has escaped detection for several years (*see below*). The agent carries a new envelope antigen, but information on this antigen and its reaction with the corresponding antibody is still lacking. This virus has been tentatively named RSV(0) to indicate its probable freedom from an associated virus. RSV(0) cannot be classified with any established subgroup of the avian tumor viruses. Its sensitivity to interference with RAV–2, a member of avian tumor virus subgroup B, is so far unexplained (*15*).

Properties of RSV(0)

Host range	*Percent embryos yielding susceptible cultures*
Type of avian embryo:	
Chicken, Hyline 934E (phenotypes C/O and C/B)	5
Chicken, RPL No. 7 (phenotype C/A)	75
Japanese quail	>95

Interference	*Interference with RSV(0) focus formation*
Interfering virus:	
RAV–1	No
RAV–2	Yes

The enhancement of RSV maturation in L–R cells after RAV infection represents a case of phenotypic mixing (*19*). Phenotypic mixing is common among avian tumor viruses (*33*) and low levels of a virus with the characteristic host range of RSV(0) have indeed been detected in several predominantly helper-controlled stocks of RSV (*15*).

A cooperative effect of RAV on RSV distinct from the helper function is the enhancement of plating efficiency, which is seen when a subgroup B RSV is inoculated into a culture which is preinfected with a subgroup A RAV (*7*, *21*, *22*). This enhancement of subgroup B RSV seems to result from an increased rate of adsorption of the enhanced virus to the preinfected cells. The crucial step leading to the increased adsorption rate of subgroup B RSV occurs *in vitro* and involves the reaction of an as yet unidentified substance released from the preinfected cells with the superinfecting subgroup B RSV (*34*).

ANTAGONISTIC INTERACTIONS OF SARCOMA AND ASSOCIATED VIRUSES

Preinfection of chicken cells with RAV induces resistance to later challenge with RSV (*1*, *7*, *21*, *22*). This viral antagonism is due to interference with the penetration of the challenge virus (*35*). Among avian tumor viruses the reaction is subgroup-specific and requires immunological cross reaction between the envelope antigens of the interacting viruses (*7*). Interference is seen between RAVs as well as between RAV and RSV, but it is detected more readily in the latter combination. The ability of associated viruses to interfere with the infection and oncogenicity initiated by the principal virus is an important property aiding in the detection of these associated agents. RAV was found in this manner, and it appears that such interfering, associated viruses are common in RNA tumor virus stocks. They are present in preparations of avian myeloblastosis virus and murine leukemia viruses (*11–13*). Inadvertent selection for these agents may explain the loss of leukemogenic activity observed after a prolonged tissue culture passage of leukemia viruses (*13*, *36*).

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Satellite Viruses, Helper Viruses, and Synergistic Action—Discussion

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DETECTION of adeno-associated virus (AAV) in cell-free lysates currently depends mainly on morphologic recognition in the electron microscope (*1–4*), any of several immunological reactions (*3, 5–8*), or on inhibition of adenovirus replication (*9–11*). Table 1 lists these detection methods in an approximate order of their sensitivity. The figures listed in the right column are approximations to indicate *order of magnitude* sensitivity relationships between some of the methods used to detect a strain of AAV type 1 and a strain of AAV type 2. In our experiments both AAV strains gave crude antigen titers of about 1:10 in the microtitration complement-fixation (CF) assay. These same suspensions could not be diluted beyond about 1:200 and still show sufficient numbers of particles when negatively stained and examined in the electron microscope. A modified precipitin reaction employing sedimentation of antigen-antibody complexes over a steep CsCl density gradient permitted detection of AAV type 2 at about a 10^{-3} dilution. Suspensions of AAV types 1 and 2, when diluted to 10^{-6}, inoculated on cell cultures with adenovirus 12, incubated for 40–44 hours, fixed and stained with fluorescing antibody, gave sufficient numbers of fluorescing foci for easy detection.

The high sensitivity of the fluorescence focus assay system for AAV detection is probably explained by the fact that 1) a cycle of virus growth is allowed, thus giving a large "biological amplification" before fluorescing antibody is applied; and 2) AAV antigen remains highly localized in the nuclei of individual infected cells (*see* fig. 1). The most serious limitation of the technique is that high-titered antisera to purified AAV's must be prepared against the four known antigenic types.

In the immunization-complement fixation method (*12*) another amplification principal is employed; *viz.*, AAV antigens in such minute quantities as to be undetectable by direct CF tests, when injected into animals, will induce detectable AAV antibody. Antibody production is then detected in CF reactions by using purified known AAV antigens. The relative sensitivity of this procedure has not been reported, but it is probably greater than detection of CF antigen directly.

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[1] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

TABLE 1.—Estimates on the relative sensitivity of various methods for detecting adeno-associated virus (AAV)

AAV detection technique	Highest virus dilution giving positive result (order of magnitude approximation)
Complement fixation	10^{-1}
Animal immunization, then CF detection of antibody	Probably $>10^{-1}$
Electron microscopy	10^{-2}
Precipitin reaction	10^{-3}
Fluorescing focus	10^{-6}
Adenovirus suppression	Unknown

Adenovirus suppression has also been used as a means of recognizing AAV. To be most useful as a detection method, one must assume 1) that all AAV strains inhibit the replication of adenovirus; and 2) low concentrations of AAV cause substantial adenovirus growth inhibition in a test system. Although these assumptions are not now certain, the clearly suppressive effect of some AAV strains on adenovirus replication suggests that this may be a potentially valuable method for detecting new AAV strains.

The fluorescence focus detection system, which is both sensitive and practical, is probably a good choice for routine recognition of *known* AAV strains. However, morphologic recognition by electron microscopy remains an important method for detecting new AAV types for which no antisera are available.

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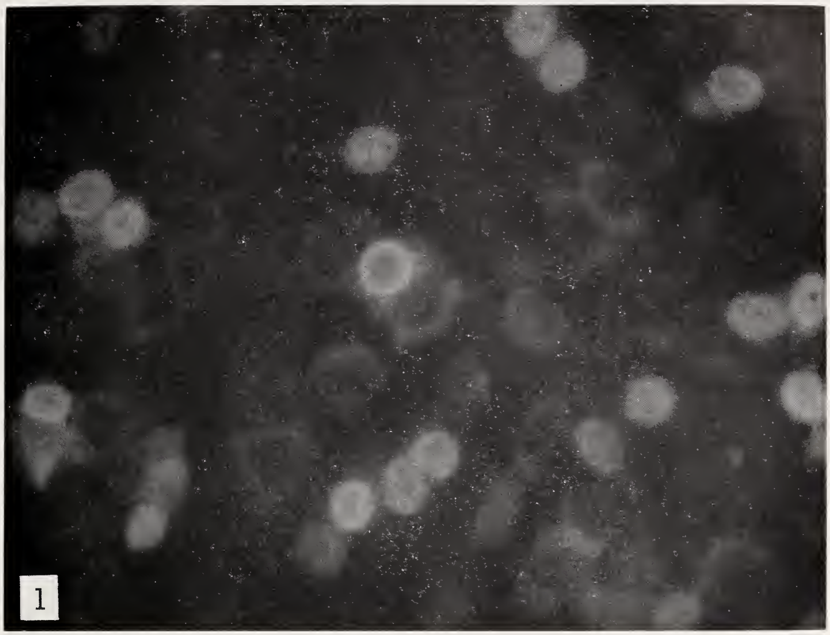

FIGURE 1.—Fluorescence micrograph showing human amnion cells doubly infected with adenovirus-associated virus (AAV) and adenovirus 12, fixed and stained to show AAV antigens only. *Note* localization of antisera in the nuclei.

Effect of Adeno-Associated Viruses on the Oncogenicity of Adenovirus 12

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I thought it might be of interest to tell this group about the effect of adeno-associated viruses (AAV) on the oncongenicity of the strongly oncogenic human adenovirus 12.

Dr. Kendall Smith, Mrs. Peters, and I investigated this point, and in two separate experiments inoculated groups of newborn hamsters with an adenovirus 12 preparation that was previously shown to be free of AAV, and also that same preparation to which AAV was added.

I think you can clearly see from the data in table 1 that the adeno-associated virus had a partially inhibitory effect on the oncogenicity of the adenovirus 12, not only producing fewer tumors but also increasing the latent period by some 13–16 days.

It would appear, therefore, that the oncogenicity of an adenovirus preparation cannot be completely evaluated without knowing whether the preparation contains AAV.

Table 1.—Effect of adeno-associated viruses on the oncogenicity of human adenovirus 12

Group	Inoculum	Number of hamsters with tumors / Number inoculated and surviving beyond 14 days	Animals with tumors (%)	Latent period (days)
A				
Experiment 1	Adenovirus 12	19/31	61.3	33
Experiment 2		7/12	58.3	34
Total		26/43	60.5	
B				
Experiment 1	Adenovirus 12 and adeno-associated viruses	10/32	31.3	47
Experiment 2		2/26	7.7	50
Total		12/58	19.0	

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[1] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

GENERAL DISCUSSION

Dr. K. Habel: It is comforting to know that some of these things work against each other.

The papers by Dr. Rapp and the two discussants are now open for general questions or comments.

Dr. J. Melnick: I would like to address this to Dr. Smith. I think he has shown clearly the differential sensitivity of the methods as he has used them, but I think he ought to divide his table 1 into 1) physical or chemical assay systems and 2) biological assay systems. If one uses a combination of systems, as he did in his immunofluorescent assay, which is in part a biological system (because the virus is going through a cycle of multiplication), the sensitivity is increased and all methods would have comparable sensitivity.

The sensitivity of the electron microscopic counting procedure increased enormously if one diluted out the satellite virus to the point where it contained a single infectious unit, replicated it in culture with the aid of its helper virus, and then looked at the culture fluid. One would find the same endpoint as by immunofluorescence. The same could be done with complement fixation as the detection method.

Dr. Habel: The importance of the amplification system is certainly true for any technique that will be used for eventual demonstration.

Dr. K. O. Smith: We have done just this with adenovirus type 12, which we have found to be the most efficient helper among four adenoviruses that we tested with adeno-associated viruses. We find that one–two cycles, requiring 48–72 hours, give a tremendous amplification for electron microscopy. Certainly if you want to throw this amplification figure in, then the sensitivity of the systems would be pretty nearly the same.

Dr. B. A. Rubin: Dr. Smith, does the adeno-12 act as an indicator culture for all types equally?

Dr. Smith: I'm sorry we don't have sufficient data for types 3 and 4 to know whether adeno-12 is a good helper with these two. But we have found that type-12 adenovirus is the best for AAV types 1 and 2. We can get good sensitivity in our titrations using adeno-12 as a helper, titrating AAV types 3 and 4, but we don't know whether we get high yields of these viruses.

Dr. M. M. Sigel: As Alice said, "things are getting curiouser and curiouser." Dr. Rapp, would you define for us, who are not working with helpers or satellites, the distinction which you have been very careful to make about satellites and helpers. Dr. Smith spoke just now about the adeno-associated viruses as a helper. So would you delineate between helpers and satellites? I know one of them is a laboratory phenomenon and one is a real phenomenon, but are there any more meaningful differences?

Dr. F. Rapp: I do not think Dr. Smith referred to the adeno-associated satellite virus as a helper. It is the adenovirus that helps the satellite virus. Reciprocal-complementation does not appear to exist for this system, according to the published reports.

Some investigators are calling these small particles "satellites" because they resemble the tobacco-necrosis satellite system. As in that system, the adeno-associated satellite virus is morphologically and serologically different from the parent adenovirus.

Dr. Melnick: I know terminology is confusing, particularly in this field. Dr. Vogt, in defining an associated virus, indicated that it is structurally related to the principal virus in the system.

For this reason we prefer to continue with the English workers' definition of the satellite viruses as those defective viruses morphologically and antigenically distinct from the helper viruses that let them grow. It seems appropriate to use the terms first established by the British workers for their satellite systems and reserve the term "associated virus" for the way it is being used by those working with the Rous—as one that is structurally identified with the virus that they help in replication.

Dr. Habel: But this association is not so true with adeno and SV40?

Dr. Melnick: That's right. Because they are often found together, SV40 might be considered an adeno-associated virus. Parainfluenza SV5 is another adeno-associated virus. The term is loose when used in this way, but the Rous people use it in a very precise sense.

Dr. Habel: What are you suggesting for the adeno SV40?

Dr. Melnick: For the so-called hybrid particle? The term that we have been using is descriptive, namely, of a particle that aids the replication of adenovirus, the second particle in the population. For this reason Dr. Rapp had the word "PARA" on his text-figure 2.

It has loosely been called a hybrid. This too is a misnomer, because the particles are not hybrids in the conventional sense. There are two kinds of particles present in the virus population: One is a true adenovirus and the other is the PARA particle.

Dr. Habel: Are you still pitching for the "PARA" term?

Dr. Melnick: This is what it is, a particle that aids replication of adenovirus.

While I have the floor, I would like to point out that Dr. Rapp included in his discussion a finding that may have relevance in vaccine production; namely, that simian adenoviruses act as helpers for human adenoviruses in green monkey kidney culture.

Some of the simian adenoviruses are tainted with oncogenicity, but others are not. Because monkey kidney cells have been cleared by Public Health Service regulations for growth of vaccines suitable for human use, and because many simian adenoviruses have no oncogenicity that anybody has been able to detect, might it not be possible to use monkey kidney cells with nononcogenic simian adenoviruses as the medium or substrate in which nononcogenic simian adenoviruses are grown for vaccine production? This would allow us to use adenoviruses that would not otherwise grow in monkey kidney cells.

Dr. Habel: It would certainly be nice to be able to use these to our advantage instead of our disadvantage.

Dr. Rapp, could you give us a little more information concerning these other simian agents that can act the same as SV40 with adeno growing in monkey kidney?

Dr. Rapp: Some years ago we tested a number of other human viruses that were able to grow in the nucleus of green monkey kidney cells to determine whether they were able to enhance the replication of human adenoviruses. Of the viruses tested, herpes simplex virus (a DNA virus) and measles virus (an RNA virus) failed to enhance the replication of the human adenovirus. We then decided to determine whether other simian agents could substitute for simian papovavirus SV40 to enhance the replication of the human adenoviruses in green monkey kidney cells.

The two viruses we chose to test were simian adenoviruses SV15 and SA–7. Coinfection of the simian cells with either of these viruses and a human adenovirus enabled the human virus to replicate as effectively in these cells as if SV40 were present. It was also possible to reduce the latent period of the human adenovirus by preinfecting the cells with either of the simian viruses.

We then tested whether the human adenoviruses could, in turn, help the replication of the simian viruses in human cells. However, both the simian viruses replicated fairly effectively in human embryonic kidney cells, and coinfection of the cells with human adenoviruses did not enhance the titers of the simian or human viruses.

These results again point up the fact that we must carefully define the virus and cell system when discussing the defectiveness of viruses. Although Dr. Huebner has mentioned that most defective viruses can probably multiply without help if the proper cell is found, I do not think we can state categorically that every defective virus can replicate unilaterally in the proper environment. There may indeed be truly defective viruses that are missing a piece of their genome. If they have no way to regain this by recombining with a piece of DNA that carries the missing information, they may be unable to replicate in any cell unless a helper virus is present. This may well be the situation with the defective SV40 determinants we are studying in the adenovirus-SV40 hybrid populations.

Dr. F. Deinhardt: I have two questions for Dr. Vogt. In your presentation you referred in a general way to Rous sarcoma virus as being defective. Did you want to imply that all strains of Rous sarcoma virus, including the Schmidt-Ruppin strain, have to be considered defective? What are the characteristics of the virus recovered from "nonproducer," or now called "L–R" cells, after passage in Japanese quail or susceptible chicken cells (PL-line 7)? Is this virus fully infectious for randombred chickens? Does it induce tumors *in vivo* or cell transformation *in vitro?*

Dr. P. K. Vogt: I will answer the second question first. The properties of RSV(0) remain the same regardless of passage, so even if passaged through quail, they have this characteristic low restricted infectivity in the chicken. The defectiveness seems to be a relative rather than an absolute phenomenon. Even the Bryan high-titer strain can no longer be considered as absolutely defective. The Schmidt-Ruppin strain is a good example of a virus which seems to be not defective at all.

Dr. G. Yerganian: I would ask the panel to discuss more fully the role of the use of heterokaryons for the completion of viral formation, principally on the following matter:

The use of heterokaryons will undoubtedly increase. However, one does use a UV-inactivated myxovirus which on not all occasions is fully inactivated, and consequently one might have interferon aspects or total interference with the completion of viral formation.

Secondly, would the heterokaryon be better replaced by a viable hybrid which can be carried as a cell line and used routinely as a subline when needed?

Dr. Habel: Dr. Gerber is planning to comment on this in his forthcoming discussion.

Viral Oncogenicity and Viral Vaccines—General Comments

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THE seven prime papers and an equal number of ancillary discussional presentations have stressed this afternoon a number of most fascinating facets of some very important problems. I can only wish I had enough time to comment on these in detail to the extent they indubitably merit, but with the temporal limitation which you have just heard imposed on me, restraint is in order. Consequently, I shall restrict my remarks to what is, in a way, a longitudinal theme that has pervaded much of the discussion. I refer to the use of human cells in culture for the propagation of viruses for vaccine purposes and the corollary problem of the possible presence of viruses of the human heritage as contaminants of such cell lines.

Dr. Melnick restated the definition of viral latency which was agreed to at the Madison Conference 10 years ago: namely, that "An inapparent infection is a primary infection evolving without symptoms and usually leaving a lasting immunity. Regardless of whether the primary infection evolves into a disease or remains inapparent, the virus in some cases may persist. If the virus persists in the absence of symptoms, the infection is *latent.* In a latent infection, the virus might or might not be in a proviral or in a vegetative state, and virus particles might or might not be produced."

Now, one of the evidences of latency of a virus in a host can be taken as the persistence—and I stress persistence—of a *high* antibody titer. This point was brought into question by Dr. Franks with reference to the serologic studies of Dr. Gerber, who found that individuals who had received inactivated poliovirus vaccines had high and persisting antibody levels to SV40, although the antibody levels to poliovirus, initially high, had declined. Similar instances in which persisting high antibody levels are ascribed to a latent infection are described with other viruses.

About 40 years ago, Levaditi applied the term "auto-sterilizable neuroinfections" to a phenomenon which he observed in rabbits inoculated with

Herpesvirus hominis. Certain animals inoculated with strains of this virus would subsequently die of an encephalitis, but no virus could be recovered from their brains.

In recent years, this matter has been pursued a little further. For example, Good and Campbell subjected rabbits recovered from a herpetic infection to anaphylactic shock and about one-third of the animals so treated responded some days later with a herpetic exacerbation, encephalitic manifestations occurring in some of the animals. A situation analogous to that in man was provided by the work of Schmidt and Rasmussen, who similarly activated a quiescent herpetic infection in rabbits by administration of an immunosuppressive agent.

Such activation of a latent virus is not unique or restricted to the *Herpesvirus group.* Thus, similar experimental activation of a virus was effected by Soave and his co-workers, who, by immunosuppressive measures, were able to bring about fatal infections in guinea pigs which had originally survived infection with rabies virus. Along similar lines, my associates and I have observed that mice which survive intracerebral inoculation with field strains of rabies virus will, when killed weeks or even months later, show the presence of fluorescein-stainable rabies antigen in the brain, although no virus can be recovered from this organ. This illustrates, incidentally, the value of the fluorescent antibody technique, which appears for some purposes, as Dr. Rightsel remarked, to be a procedure of choice.

Dr. Melnick mentioned several agents as examples of long-persisting viruses: *e.g.*, varicella, cytomegalovirus, herpesvirus, and a relatively recent newcomer to this list, rubella (German measles) virus. We might now, as I will bring out in a moment, probably add rubeola (measles) virus.

Detection of the presence of a virus in human tissues or in cultures of human cells would depend upon the degree of development or maturation of that particular agent during replication. If the virus attains the fully vegetative stage and produces cytopathic effects, its presence becomes readily apparent. If, on the other hand, the virus multiplies without any expression of cytopathogenicity, it becomes necessary to look for other evidence of viral expression, such as the occurrence of soluble antigens, group-specific antigens, or hemagglutinins. However, there is a corollary situation, one in which the agent replicates and its presence cannot be readily detected except by techniques which are not often applied. I have in mind the use of electron microscopy for the detection of the possible presence of virus-like particles, as has been mentioned several times this afternoon, and fluorescent antibody techniques for the detection of fluorescein-stainable viral antigens. These two techniques, especially when used in conjunction, serve to reveal the presence of viruses which are defective in the sense that they lack the infective capacity.

Dr. Melnick mentioned the recent addition of Dawson's inclusion encephalitis to the list of diseases which represent a persisting, latent infection by a viral agent. This disease, now renamed "subacute sclerosing panencephalitis" by many workers, has recently been shown to be due to the measles virus or to a virus so closely similar immunologically as to be indistinguishable from it.

The three patients listed in table 1 were all diagnosed clinically to have subacute sclerosing panencephalitis and all showed personality changes with a rapidly deteriorating mental state. In each case a brain biopsy was done and examination by electron microscopy showed the presence of intranuclear and cytoplasmic inclusions which contained tubular filaments from 170–200 A in diameter, a picture suggestive of a myxovirus infection.

When immunofluorescent microscopy was done by the indirect method using measles antisera, bright-staining intranuclear and cytoplasmic particles and masses resembling inclusions were seen. No staining occurred when distemper or herpesvirus immune sera were used, and staining with measles immune serum could be entirely or almost entirely eliminated when the serum was absorbed with measles virus. Three similar cases were described by Connolly *et al.* several weeks before our report (July 1967) and table 2 summarizes the findings in these six cases, plus one which was reported by Dayan and one we are currently studying. As is seen, in every instance in which the fluorescent antibody technique was applied, staining of brain cells occurred in the presence of measles immune serum and, in addition, *high* or rising levels of measles antibody were found in the serum. Interestingly enough, high levels of antibody were also encountered in the cerebrospinal fluid.

This past August, Legg reported that complement-fixing and hemagglutination-inhibiting antibodies to measles virus were found in the serum of 22 patients with subacute sclerosing panencephalitis in a significantly higher titer than was found in control individuals.

It is of interest that in the patients reported by Connolly *et al.*, and by our group as well as by others, the encephalitic manifestations occurred from about 4–17 years after the initial attack of measles; ostensibly the virus persists, to produce a subacute or chronic disease years later. In this connection we might mention the interesting findings of John Adams and his associates, of the School of Medicine, University of California at Los Angeles, who have reported finding very high levels of measles antibody in patients with multiple sclerosis.

In summary, therefore, the available evidence suggests that Dawson's inclusion encephalitis or, to use the more recently adopted terminology, subacute sclerosing panencephalitis, is caused by measles virus and arises as a consequence of reactivation of a long latent infection. The presence of a virus could not be detected by inoculation into tissue culture systems or animal hosts, but was suggested by electron microscopy; and by fluorescent antibody methods it was possible to identify the virus as that of measles, or at least an agent so similar immunologically as to be indistinguishable. This illustrates that a virus may be present and cannot be recovered,

Table 1.—Results of immunofluorescence studies on three patients with subacute inclusion body encephalitis related to microscopic findings

Clinical and laboratory aspects	Patient 1, F.L.	Patient 2, J.C.	Patient 3, J.U.
Age and sex	7-year male	12-year male	8-year male.
Illness:			
Date of onset	December 1965	April 1966	February 1967.
Clinical signs	Personality change; progressive deterioration to comatose, decorticate state.	Several generalized seizures in 9-month period; rapid deterioration to semicomatose, quadraparatic state over 3 weeks.	Insidious personality change, myoclonic jerks, mild intellectual deterioration.
Duration at brain biopsy	9 months	10 months	3 months.
Light microscopy:			
Eosinophilic inclusions	Inclusions present, intranuclear and cytoplasmic; in neurons, astrocytes, and oligodendrocytes.	Numerous large inclusions, intranuclear and cytoplasmic; in neurons, astrocytes, and oligodendrocytes.	Inclusions present; smaller and less numerous than in cases 1 and 2.
Electron microscopy	Intranuclear and cytoplasmic inclusions containing tubular filaments (170–200 A in diameter).	Numerous intranuclear and cytoplasmic inclusions containing tubular filaments.	Inclusions with tubular structures present; smaller and less numerous.
Immunofluorescent microscopy:			
Measles immune serums	Bright-staining intranuclear and cytoplasmic foci resembling inclusions; some neurons with staining of axons and/or dendrites.	Many cells with bright-staining particles and large inclusion-like masses, intranuclear and cytoplasmic; many cells appear filled with antigen.	Few, very small, dispersed, brightly staining particles, inclusion-like foci; few cells involved.
Distemper immune serum	No staining	No staining.	
Herpesvirus immune serum	No staining	No staining	
Normal serum	No staining	No staining	No staining.
Measles immune serum absorbed with measles virus.	No staining	Staining greatly reduced	

but can be detected by the application of electron microscopy and immunofluorescent techniques used in conjunction. It also points up what Dr. Huebner mentioned earlier, namely that under certain environmental conditions any virus may exhibit characteristics or properties associated with defectiveness.

TABLE 2.—Summary of immunologic findings implicating measles virus in cases of subacute inclusion body encephalitis

Patient	Age	Sex	Date of onset	Duration at biopsy or autopsy (months)	Fluorescent staining of brain cells with measles immune serum	High and/or rising titer of measles antibody	
						Serum	CSF
1 F.L.*	7	M	Dec. 1965	9	Present	Rising	High
2 J.C.*	12	M	Apr. 1966	10	Present	Rising	nt§
3 J.U.	8	M	Feb. 1967	3	Present	Rising	High
4 J.P.	6	F	May 1967	3	nt§	High	High
Connolly 1†	12	M	Apr. 1965	17	Present	Rising	High
Connolly 2†	16	M	June 1965	6	Present	High	High
Connolly 3†	17	M	Oct. 1965	3	Present	High	High
Dayan 1‡	5 mo	M	?	10	nt§	High	nt§

*Patients previously described by Freeman *et al*. Lancet 2: 129–131, July 15, 1967.

†Patients described by Connolly *et al.* Lancet 1: 542–544, March 11, 1967.

‡Patient described by Dayan *et al.* Lancet 1: 980–981, May 6, 1967.

§nt=Not tested or no data.

VIRUS VACCINE PRODUCTION

Viral Oncogenicity and Viral Vaccines—General Comments

Alan S. Rabson, *Pathologic Anatomy Branch, National Cancer Institute,*[1] *Bethesda, Maryland 20014*

ALTHOUGH a great deal has been said today about tumor viruses, very little has been said about the tumors they produce. Dr. Huebner has already presented a classification of oncogenic viruses and I would like to add a listing of some of the histologic types of tumors they can produce.

Some Histologic Types of Tumors Produced by Oncogenic Viruses

1. Pox viruses—
    a. Fibromas
    b. Myxomas
    c. Histiocytomas
2. Papilloma viruses—
    a. Squamous cell papillomas
    b. Squamous cell carcinomas
3. Polyoma virus—
    a. Salivary gland tumors (epithelial)
    b. Lacrimal gland tumors (epithelial)
    c. Neuroepithelial tumors of the nose
    d. Odontogenic tumors
    e. Mammary gland tumors
    f. Hair follicle tumors
    g. Epithelial thymomas
    h. Thyroid tumors (epithelial)
    i. Subcutaneous sarcomas and sarcomas of kidney
    j. Hemangioendotheliomas of liver
    k. Meningeal sarcomas
    l. Bronchiolar tumors of lung
4. Simian virus 40—
    a. Subcutaneous fibrosarcomas
    b. Papillary ependymonas of brain

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[1] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Some Histologic Types of Tumors Produced by Oncogenic Viruses—Continued

5. Adenoviruses—
   "Undifferentiated malignant tumors" at inoculation site
6. Rous sarcoma virus-avian leukoisis complex—
   a. Subcutaneous sarcomas (fibrosarcoma and myxosarcoma)
   b. Leukemia (lymphocytic, myelocytic, and erythroid)
   c. Papillary adenocarcinomas and "nephroblastomas" of kidney
7. Murine sarcoma-leukosis complex—
   a. Subcutaneous sarcomas (rhabdomyosarcomas, fibrosarcomas, and osteosarcomas)
   b. Leukemia (lymphocytic, myelocytic, and erythroid)
   c. Lymphosarcomas
8. Mammary tumor virus—
   Mammary gland tumors

As you can see, the oncogenic viruses produce a wide variety of tumors including both benign and malignant mesenchymal and epithelial neoplasms. Nevertheless, it should be pointed out that there are a number of histologic types of tumors found in man and in animals that have never been produced by inoculation of any of the known oncogenic viruses. Some of these are listed as follows:

1. Carcinomas of stomach, intestine, liver, and pancreas.
2. Carcinomas of uterus, ovaries, urinary bladder, testes, and prostate gland.
3. Malignant melanomas.
4. Functional endocrine tumors.
5. Ocular tumors.

In the follow-up of individuals receiving viral vaccines prepared in a variety of types of cell cultures of both human and animal origin, an awareness of the types of tumors produced in animals by the known oncogenic viruses may be of importance in evaluation of the data.

Viral Oncogenicity and Viral Vaccines—General Comments

PAUL GERBER, *Laboratory of Viral Immunology, Division of Biologics Standards,*[1] *Bethesda, Maryland 20014*

ONE of the important problems facing this Conference is the potential use of diploid cell lines in vaccine production. In this regard, some of the points mentioned by Dr. Melnick and Dr. Henle assume particular significance.

The first of these I should like to comment on is the use of cocultivation or cell fusion of test cells with suitable indicator cells for the detection of a hypothetical viral genome.

In the virus-free SV40-induced hamster tumors, we observed that the presence of the whole viral genome could be detected by cocultivation of intact, viable tumor cells in direct contact with African green monkey kidney cells. This process involves a cell-to-cell transfer of subviral genetic material from the tumor to the indicator cell.

This interaction could be greatly enhanced by promoting cell fusion with Sendai virus inactivated by ultraviolet light, and in answer to Dr. Yerganian's question, in our hands no residual viral infectivity of properly inactivated Sendai virus is detectable. This method has also been applied by several workers to recover infectious Rous sarcoma virus from virus-free Rous tumors induced in mammals.

In a tumor induced by defective Rous virus, it was necessary to add a helper virus to the mixture of tumor and indicator cells to recover infectious Rous virus. Recently Huebner and co-workers have applied this approach to the rescue of the viral genome in murine sarcomas.

A sensitive indicator cell and the appropriate helper virus, of course, are needed to apply these findings to detect subviral components in diploid cell lines.

The second point I should like to discuss is the importance of electron microscopic examination of concentrated preparations of cell cultures intended for use in vaccine production.

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[1] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

As some of the previous speakers mentioned earlier, the presence of herpes-like viruses in cultures of Burkitt's lymphomas was first detected by electron microscope studies.

Recently we and other workers have found that viruses of similar morphology were present in 4 of 6 lymphocyte cell lines derived from normal blood donors. These viruses are morphologically similar to the known members of the herpes group, but have so far failed to show any antigenic relationship.

Serologic evidence indicates the widespread distribution of these agents among human and nonhuman primates. These agents appear to be closely associated with lymphoid tissue of primate origin, fail to be transmissible to other tissues, and without careful electron microscopy they would have probably remained undetected.

Despite the relatively low sensitivity of electron microscopy for virus detection, it would therefore seem essential to require periodic electron microscopic examination of diploid cell strains that are to be certified for use in vaccine production.

The last point I wish to discuss is the use of immunofluorescence tests for the detection of unknown agents in cell lines, as already alluded to by some of the previous speakers.

With the use of the cultures of lymphocyte from normal donors as a model, 5 of these 6 cell lines showed specific immunofluorescence when stained with fluoroscein-labeled, pooled γ-globulin from normal donors. In 4 of these 5 positive cell lines, herpes-like virus particles were found by electron microscope tests. This suggests the potential value of this method as an additional test for certification of diploid cell lines for vaccine production.

GENERAL DISCUSSION

Dr. H. Koprowski: I would like to add a little more about the fusion and heterokaryon formation of the virus.

With regard to the relationship between tumor viruses and cell—and here I will refer mostly to experiences with SV40—we can differentiate four classes of relationship as to production of virus progeny and mechanism of virus release.

The first is obvious. This is large production of virus in monkey and human cells, and the mechanism of virus release is by lysis.

The second class is the one to which reference was made to the interaction where virus release may occur spontaneously, but cocultivation of the transformed cells with susceptible cells leads to an increased production of the virus. Some of the SV40-transformed mouse cells fall in this class.

Within the third class of virus-cell interaction, no spontaneous virus release is observed. Virus release does not occur after cocultivation of the two cell systems, but cell fusion in the presence of Sendai virus leads to the production of viral coat protein in heterokaryon cells.

Finally, a fourth class of virus-cell interaction has not been mentioned. In this class, virus cannot be activated in the transformed cells by fusion with susceptible cells. It can be isolated, however, in cultures formed by fusion of two types of transformed cells. This class presents most interesting aspects. We should consider the possibility that a variety of deficient virus mutants is involved, which may recombine to produce infectious virus.

Dr. K. Habel: It should be added that attempts to use the cell fusion method to activate other DNA tumor viruses, polyoma virus, for instance, have been unsuccessful.

Dr. W. F. Daniels: Why hasn't uranium-labeled antibody been mentioned, or isn't it of any use?

Dr. F. Rapp: I believe Dr. Sternberger and his colleagues used uranium-labeled antibodies to demonstrate vaccinia antigen in infected cells. The method has not yet been widely applied, and this, plus the lack of time, is probably the major reason for not discussing the procedure. Also, it has yet to be demonstrated whether the uranium method is more sensitive than the method using ferritin-labeled antibodies, a procedure which has also not been discussed.

Dr. Koprowski's words were well stated, although I do not think any of us referred to the virus in the transformed cells as defective. It is true, however, that some of the transformed cell cultures, regardless of fusion attempts, have failed to release virus, even after the addition of a variety of helper viruses. For example, we have failed to isolate a defective SV40 genome from these cells with the help of adenoviruses, after fusion of the transformed cells with susceptible simian or human cells.

Dr. H. Malherbe: Dr. Lennette, was it possible to distinguish whether the antigens you detected by immunofluorescence in the subacute encephalitis cases were measles or distemper?

Dr. E. H. Lennette: Yes, conjugated distemper serum was used, and there was little or no staining, perhaps because the serum wasn't as potent as the measles serum.

On the other hand, we used the sera of the patients who were reported here, plus the serum of another one, and tested against measles virus and distemper virus; these sera had very high antibody levels, something like 16,000.

Dr. Malherbe: Against both viruses?

Dr. Lennette: Yes.

The reason for thinking this is measles is that distemper infections in man are unknown, as far as I am aware.

One other statement: In no instance with conventional tissue culture techniques were we able to recover virus from these biopsy specimens of the brain. Materials were also sent to Dr. Gidisek at the National Institutes of Health, and he, too, has been unsuccessful in isolating anything.

In one sense the virus might be considered defective measles virus, since it is not infectious. The other possibility is that it is neutralized. A great deal of antibody is bound to it. There is some suggestion that this is so because normal γ-globulin will give a faint stain in the indirect test.

Dr. J. Melnick: Regarding Dr. Lennette's work with this virus, I would like to call attention to an experience that Dr. Benyesh-Melnick recently had in examining a kidney and spleen from a young child who died of giant cell pneumonia. She looked for virus by conventional means of grinding up the tissues and subculturing, but she got a negative result. The culture of the kidney and spleen cells was carried serially for some 3 months before measles virus appeared in the culture.

Are high temperatures needed to cultivate these cells so as to make them productive, in addition to the conventional means of looking for virus?

Dr. Lennette: No, we didn't. We made a mash and used suspension in the conventional tissue cultures available in the laboratory, and the same material was used by Dr. Gidisek, not for animal inoculation. We haven't tried to carry these. Of course, we are learning something about these viruses which produce these chronic illnesses. I think in the future many of these techniques suggested here will be used.

Dr. L. Hayflick: Just one observation: I agree completely with the importance of several of the proposed tests, specifically electron microscopy, flourescent-antibody tests, and heterokaryon formation for the detection of latent viruses, several of which already have been applied to the human diploid cell populations.

However, how many of the currently acceptable primary cell populations would pass muster if these tests were applied to them?

Dr. Habel: I assume this is a hypothetical question, and in view of that I would like to thank the speakers and also the audience.

Session V

Experience With Vaccines

Chairman: H. Koprowski
Rapporteur: George Gee Jackson

Monitoring Vaccines for Human Oncogenicity

ROBERT W. MILLER,[1] *Epidemiology Branch, National Cancer Institute,[2] Bethesda, Maryland 20014*

THE discovery that simian virus 40 (SV40) was inadvertently contained in some polio vaccine used in the the past led to widespread concern that this agent, oncogenic in certain newborn laboratory animals (*1*), might also be oncogenic in man. Other viruses which are known to be oncogenic in lower species may be present in animal tissues from which human vaccines are made. The influence of these viruses on cancer occurrence in man can be evaluated through epidemiologic studies.

POLIO VACCINE

An epidemiologic evaluation (*2*) was made to determine if children who had received relatively high doses of SV40 had a different cancer experience than those who received vaccine containing little or none of this virus. As is usual, the epidemiologic study had to make the most of natural circumstances. In so doing, the resources of four Federal health agencies were involved.

Mass polio vaccination began in May 1955 with immunization of children in the first two grades of school throughout the United States. In theory, study could be made of the cancer experience of this cohort as it moved through time. In practice, the Division of Biologics Standards of the National Institutes of Health was able to tell us which of the vaccines contained viable SV40, through study of samples which had been stored there for 7 years. The Communicable Disease Center provided records for the distribution of the vaccine by manufacturer and lot number, which made it possible to classify the States as to the estimated per capita dose of SV40 in the vaccines used. The National Vital Statistics Division provided IBM cards with data abstracted from the death certificates for all persons under 20 years of age—approximately 50,000—who had died of cancer between 1950 and 1959. The Epidemiology and Biometry Branches of the National Cancer Institute then made a cohort analysis of cancer mortality among those who were 6–8 years of age in 1955 until they were 10–12 years of age in 1959. The results showed no difference in mortality patterns with respect to three levels of SV40 contained in the polio vaccine. The possibility remains that the dose of SV40 was too low, the latent period too short, or the children too old to show an oncogenic response.

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[1] The assistance of the Division of Biologics Standards, National Institutes of Health, and of Dr. Fred R. McCrumb, Jr., in providing rosters of measles-vaccinated children is gratefully acknowledged.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

A human situation, which more closely approximates the circumstances under which SV40 is oncogenic in laboratory animals, involved about 1,000 newborns who received relatively large doses of SV40-containing polio vaccine by mouth. Animal experimentation indicated, however, that SV40 was not oncogenic when administered orally (*3*). Nevertheless, a follow-up study is being made of the 1,000 newborns, and the paper by Dr. Robbins, that follows, reports on the results to date.

YELLOW-FEVER VACCINE

The yellow-fever vaccine used by the Army after July 1, 1942 was of the aqueous-base, serum-free, live-virus type produced in chick-embryo cultures. The use of chick embryos suggests the presence of viruses capable of inducing neoplasia in the avian species. The question as to whether these viruses may also be oncogenic in man is now under study by the Follow-up Agency of the National Academy of Sciences-National Research Council. The sample consists of about 3,000 white Army veterans of World War II who have died of cancer since 1950 as compared with an equal number of veterans matched with respect to year of birth and quarter-year of entry on active duty. Their military service records are being abstracted for 22 items of information including vaccination against yellow fever. Determination of the frequency of such vaccination among men who have died of specific types of cancer as compared with the controls will soon be made.

NEW VACCINES

As new vaccines were introduced, we realized the need, before Dr. Joshua Lederberg wrote of it in his weekly newspaper column (*4*), for a record-linkage system which permitted easy determination of the cancer experience among children in the years following vaccination. A national childhood cancer registry based on death-certificate information, suggested by Dr. Brian MacMahon, provides the opportunity for such record-linkage. The registry, which has been established by the National Cancer

Institute with the cooperation of the National Vital Statistics Division, contains information from the death certificates of all children who have died of cancer in the United States from 1960–64. The registry serves several purposes. An alphabetical list of names of children who have died of cancer is being prepared for matching against the names of special interest. The system has already been applied to measles vaccine prepared on canine kidney or chick-embryo culture and used in Maryland in 1961. Of the 6,939 children so vaccinated, none had died of cancer within the State through 1964. One to two deaths from cancer were expected in a group of this size over a period of 3 years. We hope to extend the study soon through 1966 and across the whole country (to detect cancer mortality among children who have moved from the State in which they were vaccinated). Thus far, through a simple, quick procedure, reassurance has been obtained that there was no early excess of cancer mortality among children who received this vaccine. We intend to examine other vaccines and other agents for an effect, either beneficial or detrimental, on cancer experience.

SUMMARY

The experience with polio vaccine containing simian virus 40 (SV40), an agent which is oncogenic in newborn hamsters, has led to the establishment of a record-linkage system which permits rapid, easy determination of subsequent cancer mortality among children receiving new vaccines. The procedure involves matching lists of vaccinees against a national registry of cancer deaths among U.S. children, beginning in 1960. The first application, which concerned 6,939 children immunized against measles in 1961, showed that no cancer deaths had occurred through 1964.

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Monitoring Vaccines for Human Oncogenicity—Discussion

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THROUGHOUT this Conference, we have been concerned with the possible contamination of vaccines with oncogenic viruses. The fact is that a great many people have received, either by inoculation or by mouth, viruses with oncogenic properties, such as SV40, the avian leukosis viruses, and no one knows how many others. As has been stated repeatedly, we can never say that any cell culture is completely free of contaminating viruses, since we find only those agents that can be revealed by the methods we are using. The theoretical possibilities are almost infinite and one must recognize that whenever a live vaccine, or for that matter an inactivated one, is administered there is some potential risk. Although we all believe this risk to be absent or negligible, its true dimensions can only be determined by observation.

Dr. Miller has presented reassuring data concerning the experience to date of persons who received SV40-contaminated poliovirus vaccine, measles vaccine, or vaccines presumed to be contaminated with the avian leukosis viruses.

Our own experience, to which Dr. Miller alluded, concerns a group of approximately 1,000 children who received oral live poliovirus vaccine within the first 3 days of life. About two-thirds of them received vaccine containing approximately $10^{4.5}$ TCID50/ml of SV40 virus. An additional 150 newborns were given intramuscular injections of rather large doses of inactivated poliovirus vaccines, later found to be contaminated with live SV40, in a concentration of about 10 TCID50/ml.

Needless to say, this situation is uniquely disturbing because of the young age of the children who were given the SV40 virus. And as has been pointed out repeatedly, certain oncogenic viruses only produce tumors in experimental animals when inoculated in the newborn period. Had we inadvertently reproduced this experimental model?

Now, as an aside, I will point out that rodents and people are very different, thank goodness, and I don't think we can immediately translate. But nonetheless, it was an unusual situation.

An effort has been made to determine the mortality experience of these children who are now 6–8 years of age. This study has been conducted by Dr. Martha Lepow in collaboration with Dr. Charles R. Stark, of the Epidemiology Branch, National Cancer Institute. Extensive information was obtained on each child which would permit follow-up.

The study children came from a highly mobile, low socioeconomic, largely nonwhite population. Although it has not been easy to maintain contact, every possible means has been employed to do so. We have been able to follow approximately 80%. Mortality has been determined by comparing the names on the study roster with the death registers of the State Department of Health of Ohio and other States where the study children are known to reside.

The experience in the study children is compared with the mortality rates for the general population of the appropriate age utilizing a modified life-table procedure. No detailed results can be presented at this time. However, analyses of the data available thus far have not revealed any excess mortality in the study population. This is total mortality, not just cancer.

Obviously, with such a limited study group, small differences in total mortality would not be detected. However, it would be possible to detect a peculiar preponderance of a particular cause of death, and it is somewhat comforting that nothing of note has been discovered so far. It is planned to continue to follow these children indefinitely.

In conclusion, I should like to emphasize once again the importance of the kind of surveillance that Dr. Miller and his group are attempting to conduct. Although their emphasis is upon cancer, other complications which might appear after an interval of many years should also be watched for. It is not an easy task, and it is made more difficult by the great mobility of our population, the inadequate staffing and support of many of our State offices of vital statistics, the imprecise reporting of causes of death, the decline in autopsy rates in some parts of the country, and the relative indifference existing among the public health and medical professions to some of these problems. It is hoped that, as our technology improves and as awareness increases of the potential hazards of many environmental factors, of which vaccines are one, we will be able to develop and maintain better surveillance procedures such as those Dr. Miller is attempting in one limited area.

It might also be suggested that as we develop vaccines—and this, Dr. Miller is obviously doing—we plan from the beginning to conduct the appropriate follow-up procedures. Furthermore, I suggest that in determining what vaccines we will release we attempt to utilize more than one cell source so that we have something to compare to something.

Dr. Miller's study of SV40 was really made possible because he could identify lots of vaccine that had differing concentrations of SV40. Otherwise, he would have had great difficulty in making any reasonable or meaningful comparison.

If we vaccinate the total population of children with a vaccine produced in one cell, we do not have a very good baseline. If we utilize several different cells or lots of cells, then perhaps we will be able to make some kinds of comparison, assuming we can keep appropriate records.

GENERAL DISCUSSION

Dr. J. Furth: Just to start the discussion, I will say a few words on the latency of tumor induction. Four years after the atomic bomb was dropped, evidence was presented at a national cancer conference for the leukemogenic capacity of radiations in mice. Two outstanding experts stated that this capacity may be true for mice, but not for man. Reports on leukemia among the atomic bomb casualties began to appear about 2 years later. Now, after some 20 years, the number of new cases of leukemia is on the decline, while mortality appears excessive from other neoplasms.

Dr. B. A. Rubin: In considering the problem of yellow fever vaccines, I wonder how important it would be to take into consideration the fact that these vaccines had a known serious contaminant that resulted in many, many cases of hepatitis with many deaths and many serious sequelae in the liver. Would not this be a possible influence on what happened that would be quite independent, perhaps, of the agents that are being considered here?

Dr. R. W. Miller: First, with respect to Dr. Furth's question, 4 years *is* a short time. All we can do is say that thus far we can see nothing of concern in that short interval. But within another few months, we will be able to extend the follow-up study of the measles-vaccinated children another 2 years. Another 2 years in Hiroshima and Nagasaki did reveal at least the leukemogenic effect of exposure to atomic radiation.

With respect to Dr. Rubin's question about other contaminants in the yellow fever vaccine, yes, they could very well influence the cancer experience of the people. There is some faint suggestion from epidemiologic studies, in fact, that infectious hepatitis may actually diminish the risk of leukemia. So there is a possibility that other agents in the vaccines may either raise or lower the oncogenic capacity of the vaccines.

Dr. J. P. Fox: I would like to respond to your suggestion that we look a little bit at the matter of follow-up and particularly to support Dr. Robbin's suggestion that plans be made for follow-up studies. I am momentarily interested particularly in the possible subtle effects that measles, which has heretofore been a nearly ubiquitous experience for mankind, may have; these effects are subtle enough that they may have escaped attention. I am thinking particularly with reference to learning capacity or certain behavioral effects.

These will not show up in the mortality statistics of the National Center for Health Statistics. I believe that it is still feasible to reconstruct or set up cohorts of individuals who are of approximately the same age now who will have been protected from natural measles because of the administration of vaccine and others who will have experienced measles because they didn't get the vaccine soon enough. I realize that there are some very serious biases introduced into groups constituted in this way, but one could probably counteract these to some extent by some judicious evaluation of some of the recognizable factors that would differ between the two groups. The time to do this is now. I think this could give us information about possible adverse effects of the vaccine, and also could give us more understanding about the possible benefits of the vaccine in terms of preventing both the adult disease and problems which the disease may have created that we are not now aware of.

Dr. Miller: Dr. Robbins raised the question of effects other than cancer from vaccines. One way to simplify follow-up is through record linkage. The suggestion has repeatedly been made recently that there be established in the United States a National Death Index, a list of all deaths in the United States arranged alphabetically. So if one took a list of names of children vaccinated or any other group believed to have an unusual risk of any disease, it would be a simple matter to find out whether or not the child was alive or dead at varying intervals after the event of interest. But so far the National Death Index has not shown much life.

Dr. R. Murray: I want to endorse something that Dr. Robbins said: The desirability of having more than one propagation system for vaccines. I think most people involved in public health campaigns, particularly with polio, realize that, if anything went wrong with that vaccine earlier, there would have been no means of vaccinating the people with regard to one vaccine. Fortunately, in the case of measles, two systems came along, one a little later than the other. And we have, of course, the chick embryo and the dog. And I would hope that in the future, with all of the resources that we have now, it would be possible for each vaccine that comes along to have different systems so that we will not have all our eggs in one basket.

One other comment I would like to make is about the live polio vaccine and SV40. The impression that many people have is that SV40 has been in live polio vaccine. The fact of the matter is that it was only in the experimental vaccines prior to widespread use that this situation existed. All of the vaccine that has been distributed in the United States since the time that it has become a commercial item has been free of SV40 as determined by the tests that are applied.

Dr. P. A. Long (U.S. Department of Agriculture, Regional Poultry Research Laboratory, East Lansing, Mich.): They talk about death as one criterion for vaccine. I would like to say maybe all clinical manifestations, whether it is death or muscular dystrophy or nervous syndromes or mental health, should be evaluated in the vaccines.

Dr. F. C. Robbins: Obviously, this would be most desirable. I think Dr. Miller can speak for himself, but death makes a relatively concrete and identifiable event which is recorded. If one tracks back from that, then one can determine what the patient died from.

I would like to emphasize, though, that sitting at the other end often in the clinical situation, I am acutely aware of the inadequacies of medical reports and medical record keeping. And the statistician or epidemiologist has to use what he is given. If the basic data are inaccurate, then all his biostatistical know-how can be of little value.

I do believe that we have a major job in educating the physicians and health personnel of this country to keep decent records and even such basic records as cause of death which often is very difficult to determine from what one gets on a death certificate. Possibly, with the Regional Medical Programs which are emphasizing continuing education—the heart, cancer, stroke programs—and also through the cancer aspects of it, we will have a very good opportunity to develop an educational program for the physicians in this country and other health personnel.

Dr. Miller: Very briefly, the epidemiologist, as Dr. Robbins has indicated, has to make the best of natural circumstances. What he is suggesting is that the best be improved by developing better record systems which will provide more natural circumstances that can be effectively studied.

Dr. S. K. Gottlieb (National Cancer Institute): I wonder if Dr. Miller can develop further his point about hepatitis virus possibly protecting against leukemia.

Dr. Miller: No. The evidence is very weak, based on an unpublished epidemiologic follow-up study of veterans of World War II who appeared to have leukemia less often than would have been expected.

Dr. R. Parrott (National Institute of Allergy and Infectious Diseases): Dr. Miller, one of the real ghosts in the vaccine field is the hamster oncogenicity of certain adenoviruses which occur naturally. A number of centers have reasonably good information on the frequency with which these agents occur naturally in children and adults. Some of them occur with high frequency. Have you or will you set up an epidemiologic model that would predict the frequency with which cancer should occur if these agents which occur in nature are truly oncogenic for man?

Dr. Miller: A very significant, but little-known development is the invention of statistical techniques for detecting clustering of rare events in time and space. There are several such statistical procedures now available. One has been devised by Mr. Nathan Mantel of the National Cancer Institute. This procedure affords the opportunity to examine the distribution of childhood cancer by cell type throughout the United States. Determinations can be made easily and inexpensively as to whether there is regional clustering, or clustering in time, or both.

These studies are in progress now, not with respect to any particular agent, but if some clustering is found, it will suggest that there is something in the environment, such as an adenovirus, which is responsible.

Cells, Vaccines, and the Pursuit of Precedent

Maurice R. Hilleman, *Ph.D., D.Sc., Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pennsylvania 19486*

IT was a cold and dismal twenty-second of January, 1954, and the swirling snow formed in great drifts about the Walter Reed Army Institute of Research. On this particular day, 25 scientists of diverse discipline braved the storm to attend a special meeting (*1*) of the Armed Forces Epidemiological Board which had been called by the then President, Dr. Colin M. MacLeod. The purpose of this meeting was to provide testimony and opinion which would be basic to applied viral immunology for years to come and which remains at the very epicenter of the deliberations of this Conference.

The adenoviruses, then called RI (*2*) or AD (*3*) agents, had been discovered, and the RI viruses had been related serologically and epidemiologically to atypical pneumonia and catarrhal fever (ARD) in the Armed Forces. But two questions were posed by the group: 1) Was there need for human volunteer experiments to complete Koch's postulates? If so, Dr. John Dingle was prepared to carry them out. 2) What about vaccines? The present author was ready to proceed toward such an end.

The problem lay in the fact that the viruses then could be propagated readily only in HeLa cell cultures, with only trivial production in available primary cell cultures of human source. Under such circumstance, could the viruses be grown in HeLa cultures and the filtered extracts from such a frankly neoplastic cell source be used for experimental studies in human subjects? If not, could the human epithelial cells Dr. Wilton Earle was then establishing in serial culture be used? Further, what about hepatitis virus in cells derived from man and nourished with human serum?

The meeting lasted only 2 hours and the declaration was feeble, but the pronouncement initiated a precedent which has been expanded and extended to form current policy. It was simply stated: "Virus material grown on normal cells inactivated by formalin is acceptable for limited immunization trials." Thereby, cell cultures derived from frankly neoplastic tissues or possessing attributes of neoplasia were definitely ruled out. Normalcy was not defined, and the matter of continual passage of cells of "normal" tissue origin was avoided. Fulfillment of Koch's postulates was deemed unnecessary.

The question of use of continuous cell lines for vaccine preparation was the subject of a second conference (*1*), which was convened by the Armed Forces Epidemiological Board in Atlantic City, New Jersey, on April 12, 1959. The diversity of opinion expressed at this meeting evidently precluded any definitive conclusion but made it clear that the possible risk attending use of continuous cell lines would be regarded as too great for vaccines against diseases of only mediocre importance.

This view eventually became a rule stated in the U.S. Public Health Service Regulations for Biological Products (*4*) which clearly forbids use of continuous cell lines for manufacture of live poliovirus vaccine and for live and killed measles virus vaccines. Such a regulation has not been uniformly accepted and has been the subject of controversy which is familiar to most workers in the field (*5–7*).

Our own further involvement with cell line cultivation at the Walter Reed Army Institute of Research came with the decision to prepare a live trivalent adenovirus 3, 4, and 7 vaccine for parenteral administration (*8*). We had been able to adapt certain adenoviruses to growth in rhesus monkey kidney cell cultures, but the frequent occurrence and uncertain detectability of indigenous monkey viruses in such preparations caused us to abandon parenteral application in man.

We turned, instead, to a "normal" embryonic human intestine line which had been developed by Henle and Deinhardt (*9*). After a few passages *in vitro*, epithelial-like cells had appeared among the fibroblasts and eventually supplanted the fibroblasts. A morphologic transformation was believed to have taken place, and the culture could be carried readily on serial passage.

After 20 passages in Henle's laboratory, the culture was taken to a commercial location where it was adapted to growth in horse serum-enriched medium, replacing the human serum hitherto used. Types 3 and 4 adenoviruses were started in the human intestine cells directly from patients' secretions, and type 7 adenovirus was propagated from virus initially isolated in primary human amnion cells. Adenovirus types 3, 4, and 7, grown in the human intestine cells which had been passed 16–23 times in the horse serum-enriched medium, were used to prepare vaccine.

For virus propagation, the cells were maintained in serum-free medium. The adenovirus-infected whole cultures were collected 2 days after virus inoculation, homogenized in a blender, and passed through a sintered glass filter of medium porosity to remove intact cells. After appropriate tests for identity, safety, and sterility, the 3 viruses were mixed in equal proportion and stored frozen as trivalent vaccine in sealed glass ampuls.

The trials in man were initiated on March 2, 1956. After administrative concurrence and individual informed consent, 6 adult male volunteers of overt good health were each given intramuscularly a 1 ml amount of trivalent vaccine.

The author (MRH), who was one of the volunteers, had previously received 3 doses of killed bivalent adenovirus 4 and 7 vaccine prepared from virus grown in monkey kidney cell cultures. Volunteer MRH showed neutralizing antibody to all 3 adenovirus types prior to live virus vaccination, while the 5 remaining persons were initially devoid of antibody to one or more of the 3 serotypes.

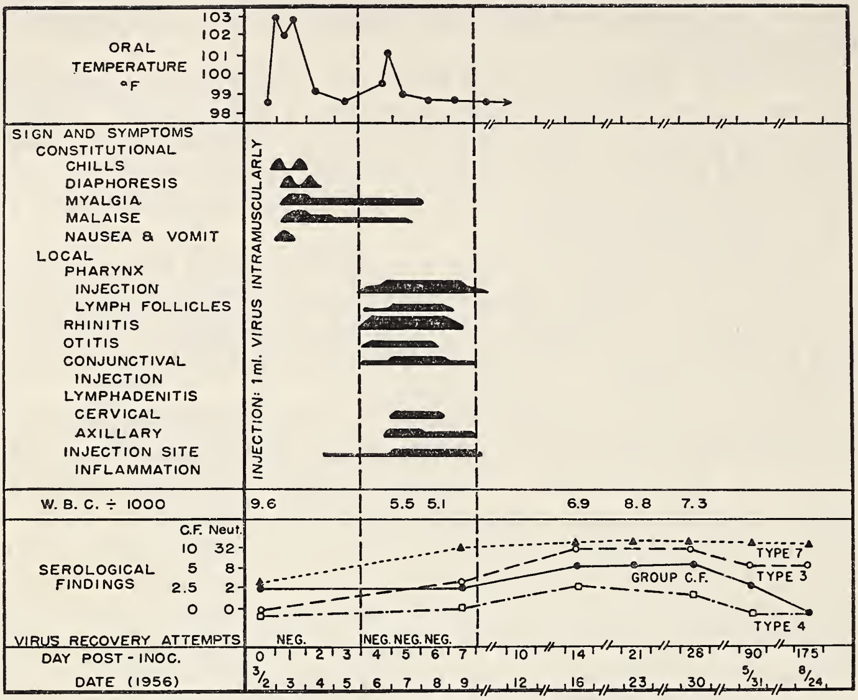
Five of the six volunteers (exclusive of MRH), reacted unexpectedly to the vaccine with a biphasic illness. Three of the six volunteers experienced an initial febrile toxic reaction with constitutional symptoms which began 7–10 hours after vaccine was given. This was believed to be due to the toxicity of the vaccine, not to infection.

The second phase of the illness began 3 or 4 days after the vaccine was administered and lasted for 4 or 5 days. It was characterized by an acute respiratory illness of mild to moderate severity which was typical of febrile catarrah or acute respiratory disease and occurred in 5 of the 6 volunteers. The chart for volunteer 2 is presented as an example in text-figure 1. Fever, constitutional symptoms, and inflammation of the mucosa of the eyes, ears, nose, and throat occurred; these were accompanied by characteristic lymphoid hyperplasia of the pharynx and conjunctiva. The inflamed eye of volunteer 3, who also displayed 2 small cystic structures in the palpebral conjunctiva, is shown in figure 1. Exudate characteristic of adenovirus infection was present on the hyperplastic lymphatic tissue in the throat of one volunteer. Cervical lymphadenopathy was common, but none of the volunteers showed signs or symptoms of spread of the inflammation to the larynx, trachea, or lower respiratory tract. The one volunteer who had previously received killed vaccine escaped all illness. The 5 volunteers had a complete and uneventful recovery, and none developed symptoms of changes indicative of hepatitis.

Adenovirus could not be recovered from the eye or throat. With 2 exceptions, the volunteers developed antibody against all adenovirus serotypes for which they were initially seronegative, and most showed an increase in amount of pre-existing antibody where it was present before vaccination. Volunteer 6, who had been previously vaccinated, showed no change in antibody against any of the 3 serotypes after inoculation of the live vaccine.

Further work with the live adenovirus vaccine was not carried out because of the high-level efficacy which had been demonstrated (*10*) in our laboratories for killed vaccine and because of the illnesses which accompanied the live virus preparation. I think the principal value of the experiments may be in the long-term follow-up. A check made in the late summer of 1967, 11½ years after administration of the live virus vaccine, revealed that all 6 volunteers were still alive and well.

Two other matters were noteworthy. First, the Henle human intestinal cell used to prepare the live virus vaccine was recently found—and Dr.



TEXT-FIGURE 1.—Clinical and laboratory findings in volunteer 2 given live trivalent adenovirus vaccine.

Coriell has discussed this (*11*)—to have been a likely HeLa cell contaminant rather than a cell of human embryonic origin. Thus, what appeared to be followed for 11½ years was a vaccine which may have been prepared from virus grown in HeLa cells. Second, and for information purposes, one of the volunteers, #6, has received repeated parenteral injections of vaccines which likely contained substantial amounts of live SV40 virus and live avian leukosis virus, influenza vaccine in Freund's adjuvant, adenovirus types 3 and 7 now recognized as oncogenic for animals, and the myriad of experimental vaccines given at Walter Reed Army Institute of Research, all without apparent untoward effect to date.

The deliberations which have taken place concerning use or nonuse of serially cultivated cells for human vaccine purposes have, I think, placed the individuals concerned into categories ranging from those who urgently itch to switch to those who would rather fight than do so and leaving an in-between group who really do not care or who do not comprehend the problem in the first place. Certainly, the more liberal point of view can be most comfortably assumed by persons with least responsibility or accountability for decision making. The more conservative view may be expected as individual responsibility increases through committee participation, and may reach its upper limit among those persons who are charged with the final responsibility for rule making.

If the debate has taken on an ecclesiastical or philosophic overtone, it should not be unexpected, since it is in an area in which the definitive information for making a judgment is clearly missing. In fact, I feel the greatest problem of all may lie in ascertaining whether there is a problem in the first place.

The simple fact is that we do not know the cause for neoplastic disease in man and we do not know if it is transmissible under any circumstance. Those workers who are most closely related to virology might argue that the analogy for viral causation of cancer in animals must surely extend to man and, hence, every effort needs to be made to prevent the transfer to the vaccine recipient of oncogenic virus, of oncogenic viral genetic material, or perhaps of neoplastic host cell genetic substance carried by a virus. Such individuals might prefer use of serially propagated cells in which the chance for new viral introductions by fresh tissues is obviated.

On the other hand, those researchers oriented toward cytology might regard neoplasia as a state of the cell itself and want to emphasize normalcy of cell with regard to karyosome, morphology, finite propagability, contact inhibition *in vitro*, and the like. Whether any of these factors has any basic importance in cancer in man or its transmission is not fully known.

Frank neoplastic cells may display no visible changes in their karyosomes (*12*), and "normal" cells, even on primary cell culture, may display aberrations such as Dr. Melnick has shown (*7*). Contact inhibition of human diploid cells, a strong criterion for normalcy, may be reduced with resulting postconfluent growth merely by substitution of galactose for glucose in the growth medium (*13*). Those researchers who place greatest emphasis on range of permissible heteroploidy of serially propagated cells may be inclined to extend the limits for acceptability as the range for such variation is recognized.

Viruses most capable of infecting a particular species may be least inclined to lead to cancer, whereas those least capable of establishing infection may be most inclined to lead to neoplasia and vice versa. There are no reliable guidelines.

In such an aura, the practical need to prevent acute infectious diseases by vaccines runs into full conflict with the theories, the "maybe's" and the "might be's" which likely can be only answered in the human species anyway. Progress must necessarily entail compromise, weighing need against potential risk.

I think the scientist, like the lawyer, is a student of precedent and reasonably follows precedent wherever possible. When the demands of a particular situation exceed the bounds of previous experience and when there are no clear-cut contraindications, the least radical departure seems to be the prudent choice and new precedent is thereby established. Whether such a course is purposely followed or not, this seems to be what happens in any case.

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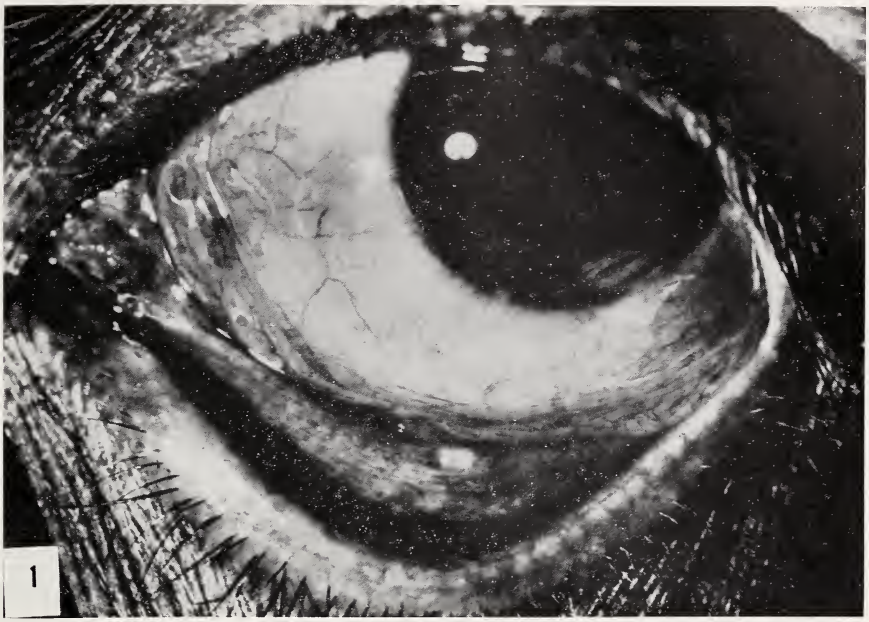

Figure 1.—Conjunctival involvement in volunteer 3.

Vaccines Produced in Heteroploid Cell Lines—Discussion

Robert N. Hull, *Lilly Research Laboratories, Indianapolis, Indiana 46206*

I would like to describe briefly one of two approaches we have followed in our investigations into the possible use of heteroploid cell lines for vaccine production. The other will be discussed later in the program. This approach to the study was patterned essentially after the proposal made by the Committee on Tissue Culture Viruses and Vaccines, as published in *Science* (*1*) several years ago.

Strain LLC–MK$_2$, established over 12 years ago in our laboratory from rhesus monkey kidney, was characterized as thoroughly as possible at both the 189th and 378th passage levels. No essential differences were noted in the cells at these two levels. A large volume of cells in the 200th passage was frozen to maintain a supply of cells between these two passage levels for years to come. In summary, about 80% of the cells contained 60–68 chromosomes with the remaining cells having either more or less. No evidence of tumorigenicity or transplantability was obtained in rhesus monkeys, newborn hamsters, or in the hamster cheek pouch. Some questionable evidence of neoplastic change was seen in the brains of cortisone-treated rats which were inoculated intracerebrally with LLC–MK$_2$ cells, but also some tubule formation was noted.

Following these characterization studies, a vaccine was prepared in the 198th passage for tests in human volunteers. The virus selected for this study was Coxsackie B-5. A new strain was isolated directly in strain LLC–MK$_2$ from a positive human stool specimen, since we did not want to use a virus which had been passaged in any other tissue culture system. The virus pool after harvest was clarified by sequential filtration through Selas XFF, C–5, and ST–1 Seitz filter pads. The postfiltration virus titer was $10^{-6.55}$/0.5 ml. Inactivation was accomplished by treatment with 1:400 dilution of formaldehyde for 6 days at 36 degrees. The vaccine and/or the harvest fluids passed all safety tests prescribed for poliomyelitis vaccine, plus additional tests which were done for the presence of adventitious agents.

The potency of the finished vaccine was compared to the live virus pool by immunization of both rats and rabbits. In rats, the live virus stimulated antibody response in the 1:64 to 1:128 range, while the vaccine produced responses ranging from 1:64 to 1:512. In rabbits, both the live virus and the vaccine produced antibody levels between 1:64 and 1:512. The finished vaccine was, therefore, fully as potent in these two species as was the starting raw virus pool.

An Ind 1571 form was filed and one subject, a man 50 years of age, was given a single 1.0 ml subcutaneous injection of vaccine in the dorsal surface of the forearm. His prevaccination antibody titer was 1:16, which rose to 1:256 three weeks after inoculation. No untoward effects were noted during 33 months of observation.

Three months later a second subject, a 38-year-old man, was given a similar injection. His preinoculation antibody titer was 1:16, and at 30 days post vaccination, it was 1:32. No adverse effects were noted during 30 months of observation.

The potency of the vaccine for experimental animals declined sharply after 2 years' storage, and the study was discontinued. It is planned, however, to produce more vaccine in strain LLC–MK$_2$, possibly with other viruses and to continue the investigation.

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GENERAL DISCUSSION

Dr. G. Yerganian: Perhaps this is a good time to reemphasize that heteroploidy of normal derivatives does not, in any way, reflect its oncogenic potential. Aside from improved cell proliferation rates, plating efficiency, tolerance to serum toxicity, etc., heteroploidy bears no direct relationship with the acquisition of neoplastic properties, spontaneously or following exposure to oncogenic viruses.

Dr. R. N. Hull: I would like to agree with you, and I add that the only reason we study chromosomes is because it is popular to do so.

Dr. M. R. Hilleman: I think this Conference has been excellent from the standpoint of defining the scientific problems relative to detecting viruses and characterizing cells. More important, one would hope that the Conference might get down to the matter of what one is going to do about these problems.

I think Dr. Murray made a very good point this morning, specifically, that one should not have all of his eggs in one basket. I presume this means all monkey cell, dog cell, chick cell, etc. Any one cell could become unacceptable overnight just the way monkey kidney suddenly went to pieces with the German experience. One should, therefore, try to certify a number of different kinds of cells that could be used in the event that one or another developed a problem.

I think also that being paralyzed out of fear for the unknown will hold back progress for a long time. If you need a cell for the purpose of controlling an important disease and there is no presently accepted cell, then one has to depart and go into the unknown to establish a new background for use.

Dr. H. von Magnus: I am sorry, I don't quite understand why this German experience has made everybody so scared about using monkey kidney tissue for vaccine. We have been told that the size of the agent is maybe 600–800 mμ and any filtration procedure you would use would remove it from the fluid. Of course, I don't want to say that it isn't a terrible experience, but I don't think it has much to do with the production of safe vaccines.

Dr. B. A. Rubin: I wonder who in Dr. von Magnus' laboratory would like to do the filtration of this vaccine.

Dr. von Magnus: It was, of course, very provocative, but this so far has been a one-time experience. We have worked for many years knowing about the casualties caused by B virus in the monkeys. I, therefore, would like to be told why this particular experience in Germany has upset everybody so much.

Dr. H. Malherbe: I understand that workers at Porton, England, have found this agent to be retained by a 340 mμ filter. Application of Black's formula of 0.64 as the ratio between electron microscope size and pore-retaining size will give us somewhere in the region of 200 mμ size for the agent, which might pass some of the procedures used for current vaccine production.

Dr. G. Barski: To answer Dr. von Magnus' statement, and in connection with the infection accidents in the two German and Yugoslav laboratories, I wanted to emphasize the following point. This accident demonstrated drastically the potential and unpredictable danger of using tissue material prepared directly from monkeys originating from areas most infected with known and unknown viruses—I mean the tropical and subtropical areas of India and Central Africa. Dozens of new virus species were discovered in these areas during the last years, and we can be sure that others will follow. We have to consider the recent accidents essentially as an additional and a dramatic proof that viral infections can burst out, in this kind of material, any time and in a quite unexpected way.

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This Conference is facing two fears concerning the use of different cells for preparation of antiviral vaccines. First, the cells are changing during long-term cultures and sometimes changing into tumorigenic cells. However, it is, so far, only a matter of speculation, if not superstition, that these cells, because they are transformed, may transmit their "malignancy" through the medium in which they grow and which is used for vaccine preparation.

The second fear, which seems to me much more real and supported by experimental evidence, is that primary cultures, especially from wild monkeys, can harbor suspect, known or unknown, agents that may be difficult or impossible to check thoroughly in the time limits and conditions proper to this kind of culture.

Yet, it is my feeling, from the papers and discussions at the meeting that we have to handle and to use for human vaccine preparation, cell cultures in which unknown and unpredictable factors are reduced to the minimum.

Dr. R. B. Stones (Chas. Pfizer and Co., Inc.): I should like to support Dr. von Magnus' view that we are becoming very alarmed over what is not a very great incident. I don't mean to diminish this tragedy that has happened in Germany in any way, but we have lived for many years now with the possibility of B virus infection arising from monkeys. I don't ever remember anybody suggesting that we should stop using monkey kidney when the risks of B virus infection were known and widely appreciated 9 or 10 years ago.

I think Dr. Barski's point that we would all like to get into a clean system is very good, but I don't think that philosophy over the use of monkey kidney should be dramatically affected by the appearance of another new simian agent.

Dr. Hilleman: I think the principal problem in this particular case was that there was no means for detecting the simian agent. The case of B virus was different, since there were good means for detecting virus by intradermal inoculations into rabbits or by cell culture.

I think also that the control measures which are applied to handle a problem tend to become too generic. Certainly, monkeys that came out of a quite different area than that in which the problem existed and which were transported by different air routes shouldn't necessarily have this same regulatory control placed on them. I presume that the ban on use of grivets will be lifted rather soon because this difference is now recognized.

Dr. L. Hayflick: I think that the position taken by Dr. von Magnus and Dr. Stones is one that could more succinctly be defined as "being happier with the devil we know than the devil we don't know." There is a very interesting extrapolation of this philosophy which says that the better we know our devil, the happier we are with him. Consequently, this year when seven people tragically died in situations where monkey kidney was used, we presume that we now know our devil a little better. Next year when, perhaps, other tragedies occur and the following year when tragedies of greater magnitude result, I presume that this will reinforce the contention that the devil we know is being known better and better and is consequently more desirable.

Dr. R. Murray: Just one or two short remarks about this monkey situation.

A number of statements have been made at various times during the meeting which lead one to believe that there is an understanding that production of vaccines in monkey kidney has entirely come to a halt. This is not so. Vaccines are still being produced in rhesus kidney and in cynomolgus kidney cultures. The only problem with the African green monkeys at the moment is that until the safety of the vaccine is resolved, there is a temporary halt in release. There is no indication that this is permanently deferred. This halt is contingent on the accumulation of experience with respect to the agent to see whether it would in fact be a danger to the vaccine itself. From what information we have gathered and what we have heard here thus far, it would appear that the system for production and testing of vaccines which is in force would probably have picked up this agent. When this matter is resolved, I think, then we can approach the question of deciding how to proceed with respect to green monkey kidney material.

Dr. T. Francis, Jr.: I was interested in Dr. Hayflick's remark about the devil. There is an old saying that to fight the devil, learn his ways. And I think what is happening here today and the last few days is an example of the way some have learned some of the ways.

I am particularly impressed with Dr. Hilleman's historical account because the second conference that he spoke of relating to continuous cell lines, I happened to be responsibile for. At that time, we drew together all the people we could find who had had any major experience with those lines.

The surprising thing was that, not only were these people unwilling, quite uniformly, to agree to the idea that these lines should be used as a basis for live vaccine, but the consensus or at least the majority opinion was that they even objected to using them for inactive vaccines.

The outcome of this was that they were not used. But one of the other features that was most impressive in the conclusion was that, not only was it a question of their being contaminated with other viruses, but many of these lines were not the lines they were said to be. They were contaminated with other lines, including HeLa cells. So when one speaks of contamination of cultures, one has to consider this constantly.

When we speak of being afraid of this or that, I would like to remind Dr. Hilleman of another experience. When he found that the SV40 was occurring in rhesus monkey kidneys and that some of this was likely to be in the polio vaccine, he argued that this was a fearful thing and that we should withdraw all polio vaccine from the market. Fortunately, the Technical Committee didn't agree.

Dr. H. Koprowski: It was Samuel Butler, I think, who in an apology for the devil said, "We have only heard one side of the case (because) God has written all the books."

Dr. Stones: I should like to come back briefly to Dr. Hilleman's point. We do have, as Dr. Murray has implied, a test system which will detect this new agent in the guinea pig. And as Dr. Hilleman knows, and as probably everybody here knows, all live poliovirus vaccines are tested in guinea pigs.

Furthermore, to reply to Dr. Hayflick's point, I don't think that anybody has ever suggested that we should abandon monkey kidney tissue for testing vaccines. And as I remember from the Opatija Conference, it was agreed there that vaccines prepared in diploid cell lines would have to be tested in monkey kidney tissue.

I would also add that the tissues that were being prepared in Germany, and with which these incidents were associated, were not being used for vaccine production. They were in fact being used for test purposes.

Dr. Koprowski: I think that some of you who discussed the Frankfurt-Marburg incident did not eyewitness the unfortunate accident. Perhaps we should ask Dr. Hennessen and Dr. Bonin, who were directly involved, whether they would like to add their observations to the discussion.

Dr. W. Hennessen: I quite agree with what Dr. von Magnus said and what Dr. Stones said. The agent which was responsible for the accidents in Germany can be found to our knowledge, and one can work in the laboratory in the presence of this agent. This is shown in all these laboratories that do the diagnostic work at the moment. But this, of course, doesn't exclude that there are better systems which do not have the potential danger of this agent.

I would like to stress what Dr. Stones said about the agent in the tissue culture. This really was in tissue culture used for testing. And this, of course, would have to be kept for other vaccines most probably, too.

Dr. Hilleman: The concern about the German monkey experience arose because there was no ready means for detecting the agent at the time the human deaths first occurred. Now that there is a test, the problem becomes much more academic.

In reply to Dr. Francis' remark, I have trouble remembering the meeting he referred to. When we first found SV40 virus in vaccines, we thought it was just another simian agent and that the simplest solution was to eliminate it without undue concern. When we found, however, that this agent was actually an oncogenic virus, it seemed to us that the vaccine lots which contained viable virus should not be used further. This judgment was fully concurred with by the National Institutes of Health and such vaccine lots were prohibited, though recall of materials actually on the market was not pursued.

Clinical Experience With Vaccines Produced in the Human Diploid Cell Line WI–38

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Clinical results of the use of live virus vaccines against poliomyelitis, adenovirus type 4, measles, and rubella produced in human diploid cell (HDC) lines are reported in this paper.

LIVE VACCINES FOR ADMINISTRATION BY ORAL ROUTE

Polio Vaccine

Live oral poliomyelitis vaccine prepared in the HDC line WI–38 has been used in the United States, Sweden, and Switzerland where, altogether, about 2,000 children have been vaccinated (*1*).

In Yugoslavia, according to data reported by Dr. Ikić (*2*), 200,000 children have been vaccinated with live polio vaccine produced in HDC line WI–38 from 1963–66.

The results thus far have been obtained in a field trial involving 5,313 children. Half of these received the Koprowski strain of type 3/WM–3/ vaccine prepared in HDC line WI–38 and the second half received the same vaccine prepared in monkey kidney tissue culture (MKTC). The groups of vaccinated children were similar. The health of the vaccinees was assessed through clinical diagnoses made by their physicians. There was no difference in the incidence and kind of diseases and symptoms recorded for groups vaccinated with the HDC line WI–38 vaccine and the groups vaccinated with the MKTC vaccine.

Special attention has been paid to infectious hepatitis because of the possibility that this virus might be present in tissue cultures of human origin. During the 12-month observation period, 6 cases of infectious hepatitis were recorded in the groups given the HDC line WI–38 vaccine and 7 cases in the two groups given the MKTC vaccines. Based on these data Dr. Ikić concluded that HDC line WI–38 does not contain infectious hepatitis virus or any other known pathogenic agent.

Data will eventually be available for the entire group of 200,000 children inoculated since 1963.

Adenovirus (Type 4) Vaccine

Dr. Bierly (*3*) reported that, during studies carried out between 1963 and 1966 inclusive, more than 42,000 individuals were fed oral, enteric-coated dose forms of type 4 adenoviruses produced in HDC line WI–38. At least 29,000 of these subjects were considered to have no measurable neutralizing antibody at the time of feeding (1 : 4–1 : 10). As Dr. Bierly concluded, administration of live, lyophilized adenovirus 4 vaccine in an enteric dose from titering more than $10^{4.2}$ in human embryo kidney selectively and silently infected the gastrointestinal tract of virtually all adults with no, or minimal, prefeeding serum-neutralizing antibody levels. This asymptomatic infection rarely, if ever, spread to contacts in personnel of military installations or prisons. A neutralizing-antibody response almost always resulted from a vaccine-induced infection of the intestinal tract.

If virus was fed the subjects 2–3 weeks before exposure to natural infection, the vaccine prevented 95% or more of type 4 adenovirus-associated acute febrile respiratory diseases (Parris Island, S.C., 1964–66). When vaccine was administered to recruits upon arrival at a base or during the first week of basic training, or, in other words, at the time of, or during, the first few days of exposure to endemic wild adenovirus 4 infections, it was less effective but still prevented 70% or more of type 4 adenovirus-associated acute respiratory diseases (Great Lakes Naval Training Center, Great Lakes, Ill., 1965, Fort Dix, N.J., 1965, 1966). The virus used in the vaccine was not detected in the respiratory tract of vaccinees.

Silent enteric infections induced by the vaccine did not prevent subsequent respiratory infection with wild adenoviruses. However, the enteric infections induced by the vaccine reduced the spread of wild adenoviruses, as compared to the circulation among unimmunized groups, and, as stated above, very obviously and significantly reduced the incidence of acute febrile respiratory disease, and to a lesser extent, afebrile respiratory disease caused by adenovirus 4.

Live, oral, type 4 adenovirus vaccine did not prevent acute respiratory disease caused by type 7 adenoviruses. The use of the type 4 vaccine, prepared by propagation on WI–38 cells, was not followed by any abnormal signs or symptoms, disease, or side effects in carefully followed and studied groups of adults. Dr. Bierly concluded that oral administration of an enteric-coated lyophilized, live adenovirus type 4 vaccine, prepared by propagation of CL68578 strain on WI–38 cells, is a simple, safe, and effective method for active immunization of adults against acute respiratory disease caused by adenovirus 4.

LIVE VACCINES FOR PARENTERAL INOCULATION

Live Measles Vaccine

At the Moscow Research Institute for Viral Preparations, we studied the HDC line WI-38 (*4*). Tests of this cell line during continuous passages fully confirmed its characteristics and revealed neither contaminating agents nor oncogenic activity. Tests conducted at the Tarasevitch State Control Institute gave similar results. Additional assay of the ultrastructure of WI-38 cells under the electron microscope did not reveal any contaminating agents (*5*).

Table 1 presents the principal steps of the control of WI-38 cells and experimental lots of measles vaccine (only special controls included).

No foreign contaminating agents in the cells or virus suspensions were revealed from these studies. The possibility of increasing vaccinal measles virus (L-16) pathogenicity during its passages through the homologous cell system (HDC line WI-38) was studied. Successive passages of L-16 strain in HDC line WI-38 have not markedly influenced its characteristics or the intensity of virus accumulation.

This virus strain, passages 1 and 16, was tested in two groups of newborn dogs by Dr. Shroit's method (*6*). The dogs were inoculated subcutaneously with 10^6 TCD50 virus and killed 12 days after inoculation. Mitoses and alteration in spleen and lymphatic nodes, and an increasing number of plasmatic cells in follicles and red pulp of spleen were observed. The dogs' sera were assayed for antibody in HI tests. No difference in the intensity of alterative changes in the two groups of dogs is evidence of similar pathogenic properties. Stability of immunogenicity was demonstrated by the similar mitotic activity in the corresponding parts of the lymphatic apparatus. Then L-16 virus was inoculated into the thalamus and lumbar part of the spinal cord of eight Cercopithecus monkeys. No case of neurotropism developed.

An experimental lot of L-16 strain vaccine was produced (after its 5th passage in HDC line WI-38) and tested in children sensitive to measles in a test parallel to that in which routine simple vaccine prepared on primary cell culture of guinea pig kidney (GPKC) was used (table 2). Thus the tests performed by different methods showed that HDC line WI-38 passages did not result in reversion to the pathogenic properties of the strain of vaccine.

In various periods during 1964-67, 22 experimental lots of live measles vaccine of different strains (Edmonston, Beckenham-20, L-16) were prepared. Some of them, after proper control, were inoculated into children who had no measles before. By 1967 we had vaccinated 182 children. Reactivity and immunogenicity of vaccines depended on the strain of virus used for the preparation of vaccines.

The vaccinated children are periodically examined for measles antibody and to check their physical condition. The observation period is from 5 months to 3 years.

Table 1.—Control of human diploid cell (HDC) WI–38. (Figures denote number of material samples/number of animals studied.)*

Materials	CPE and HA tests				Chick embryos	Animals			Interference HGP, ECHO–11	Cytology of stained preparations	Stability of karyotype	Tumorigenicity in hamsters	Neurovirulence in monkeys
	Primary cells			HDC, another strain		New-born mice	Adult mice	Guinea pigs					
	GMKC	RK	HAm										
HDC in different passage levels.	32	34	34	41	2/40	2/40	2/40	2/20	31	30	20	23/245	
Vaccine pool before centrifugation. . .	28	30	31	30	27/520	23/348	23/420	19/143				2/19	2/8

*GMKC=green monkey kidney cell; RK=rabbit kidney cell; HAm=human amnion cell; HDC=human diploid cell.

TABLE 2.—Comparative test of human diploid cell (HDC) line WI–38 and guinea pig kidney cell (GPKC) vaccines

Type of reactions	GPKC	HDC
Total number of vaccinated children	59	42
Temperature:		
0–37.5° C	6.7%	4.8%
37.5°–37.6°–38.5° C	13.5%	4.8%
Higher than 38.6° C	6.7%	2.4%
Rash	10.1%	4.8%
No reactions	73.1%	88.0%
Serological conversion	83.0%	95.2%
Mean antibody titer	62	111

Repeated serological assays, 1–3 years after vaccination, were performed on 78 children. In 86% of the children, measles antibody persisted although their titers fell fourfold. No antibody was found in 14% of the children examined. The percentage of seronegative children depended on the vaccinal strain with which these children were vaccinated. Among the vaccinees who were given more attenuated strains L–16 and Beckenham-20, 20% of the children lost their antibodies, and 7.6% among those vaccinated with less attentuated Edmonston strain. This antibody dynamics is characteristic of live measles vaccines and consistent with the results of vaccination with similar preparations produced on other substrates. Thirty-seven vaccinated children who were in contact 2 months to 2.5 years after the vaccination with persons having measles did not develop the disease, whereas 139 children in the control group under the same conditions fell ill with measles.

Clincial analysis of blood samples from 101 children from 5 months to 3 years after the vaccination revealed no pathological changes when compared to the control group of 33 children not vaccinated or vaccinated with some other measles vaccines.

One hundred and sixty-three vaccinated children were clinically observed. This group of children did not differ from those in the control group in the level and character of their diseases and in their physical condition. Thus, on the basis of laboratory tests, the vaccines prepared in HDC were free of any contaminating agents and safe for the vaccinated children. The vaccinal reactions they caused and their immunogenicity have been consistent with those of the vaccinal measles strain used.

In 1967, on the basis of our materials, we were given permission to vaccinate children to extend clinical observation of live measles vaccine produced in HDC line WI–38. A large lot of measles vaccine (strain L–16) was produced. Preliminary test of the vaccine on children sensitive to measles revealed the usual reactions for this strain (L–16). Seventy percent of the vaccinated children showed no clinical reactions. Positive seroconversion was found in 90% of the children.

Then 2,707 children from 1–7 years of age who had no measles before (according to anamenestic data) were vaccinated with this lot of vaccine. Most children were inoculated by means of jet injector, 102 children, subcutaneously, 2,557, intracutaneously; and 48 children by routine subcutaneous injection. In all cases the dose of virus used was the same. Clinical reactions were recorded on 2,184 children. There were 7.3% of the children with temperatures higher than 38.5°; 10.5% had 37.6–38.5°; 13% had 37.5° and below. There were 69.2% children without clinical reactions.

Double serum samples were taken from 448 children on the day of vaccination and 1 month later. From those observed, 35.9% had measles antibodies before vaccination and 64.1% were seronegative. Analysis of seroconversion in seronegative children revealed some differences in their reactions depending on the method of inoculation of the vaccine (table 3). Thus, after extended observation the live measles vaccine (strain L–16) strain used has shown moderate reactivity and high immunogenicity. No pathological reactions or diseases were observed in the vaccinated children.

TABLE 3.—Reactivity and immunogenicity of live measles vaccine L–16 in human diploid cell line (HDC) WI–38

Method of vaccination	Number of children vaccinated	Temperature reactions*				Immunogenicity		
		None	To 37.5°	37.6–38.5°	38.5° and up	Number of sera assayed	Seroconversion (%)	Mean antibody titer
Subcutaneous	150	69. 2	13. 0	10. 5	7. 3	56	95	314
Intracutaneous	2557					253	91	143

*Temperature reactions are given in percentages.

Dr. Ikić (in personal communication) has noted satisfactory results of vaccination of 25,000 children with live measles vaccine produced in HDC in Yugoslavia (*7*).

Live Rubella Vaccine

Dr. Plotkin (*8*) has passed rubella virus through 16 passages in HDC line WI–38, and obtained a very high yield of virus. The virus continues to give plaques in RK 13 cells and produces interference in WI–38 cells. A few children, 18 months to 3 years of age, were given the vaccine parenterally at various passage levels. The vaccinal process was observed in the children. The children remained well and were studied for longer periods.

Table 4 summarizes the clinical experience with vaccines produced in HDC in four countries. Oral inoculations of live polio vaccine into children and adenovirus 4 into adults, or parenteral inoculations of live measles

TABLE 4.—Results of extended experience with virus vaccines produced in human diploid cells (HDC) line (WI-38)

Vaccine	Author of the report	Country	Years	Inoculation method	Number of vaccinees	Age of vaccinees	Results		
							Cell substrate reactions	Vaccinal reactions	Epidemiological effect
Poliomyelitis	Dr. D. Ikić *et al.*	Yugoslavia	1964–1966	Oral	200,000	Children	None	N	+
Adenovirus type 4	Dr. Chanock *et al.*	United States	1966	Oral	42,000	Young adults	None	N	+
Measles	Dr. O. G. Andzaparidze.	USSR	1964	Parenteral	182	Children	None	N	+
	Dr. O. G. Andzaparidze.	USSR	1967	Parenteral	2,747	Children	None	N	+
	Dr. D. Ikić	Yugoslavia	1966	Parenteral	25,000	Children	None		
Rubella	Dr. S. Plotkin	United States		Parenteral	A small group of children.	Children	None	N	

and rubella vaccines into children, have not produced in the vaccinees any pathological reactions or diseases caused by the substrate used for the vaccine preparation. From these observations and the results of laboratory tests of the HDC line WI–38 we concluded that this cell system is safe.

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Vaccines Produced in Diploid Cell Lines

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THE use of well-characterized substrates is as essential for the production of virus vaccines as is a well-characterized seed virus. The problem of safety of substrates for virus vaccine production is as important as the problem of safety of seed virus. Considered from this aspect, primary cells have certain limitations. Human diploid cells (HDC) have the advantages that 1) they can be well characterized, 2) they are susceptible to a number of viruses, and 3) a number of different vaccines can be prepared in this system. This is the reason that in the past 5 years there has been a growing interest in HDC as a substrate for virus vaccine production. During this time a substantial body of evidence has been collected on HDC, on the safety of this substrate, on the titer and the genetic stability of viruses propagated in this substrate, and on the immunizing potency of vaccines prepared in this substrate (*1–11*).

SAFETY OF HUMAN DIPLOID CELLS

For almost 5 years we studied this substrate to gain knowledge on whether it harbors latent viruses, what its oncogenic activity is, if any, and whether it represents a uniform population of diploid cells of normal properties. So far, we have prepared in HDC two lots of poliomyelitis vaccine (Koprowski, live oral polio vaccine, types 1, 2, and 3) and about 10 small lots of measles vaccine, using further attenuated strains of Edmonston B–H–Z[1] and Leningrad-16 measles viruses. During this study we examined both Thompson flasks of HDC tissue cultures and virus suspensions before filtration. The materials used are listed below.

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[1] Edmonston B–H–Z is Edmonston B measles virus propagated in our laboratory. H stands for HDC and Z for Zagreb.

Materials Used in the Control of Poliomyelitis and Measles Vaccines Prepared in Human Diploid Cells

473 Thompson 2-liter flasks with human diploid cells
12,600 test tubes with tissue culture (Cercopithecus, HDC, human amnion, rabbit kidney, HeLa)
912 baby bottles with tissue culture (Cercopithecus, HDC, human amnion, rabbit kidney)
3,420 laboratory animals (mice, baby mice, hamsters, rabbits, and guinea pigs)
11,151 ml virus suspension
150 monkeys used for neurovirulence test
3,100 cells counted in metaphase
610—detailed analysis of cells—tetraploidy observed in 2–3% of cells

All tests during the 5 years of study gave negative results: no latent viruses and no oncogenic activity. The WI–38 cells represent a uniform population of diploid cells of normal properties. In addition, other tests, not mentioned in the above list, were made and also gave negative results (*11–13*). The same experiences have been reported by Dr. Andzhaparidze, who has been producing and studying measles vaccines and human diploid cells for 3 years (*14–15*). The Perkins group (*16*) has reported the same results in a several-years-long study, as have all other laboratories concerned with this subject.

VIRUS TITER IN HUMAN DIPLOID CELLS

The titer of poliovirus in HDC is nearly that obtained in monkey kidney tissue culture (MKTC). For type 1 (CHAT) poliovirus, it ranged, expressed in logs, from 7.4–8.7; for type 2 poliovirus (WI–2) from 6.7–8.2, and for type 3 (WM–3) from 6.8–8.0 in 1 ml (*11*). The titer of Edmonston B–H–Z and Leningrad-16 measles viruses in our laboratory was between 4.5 and 5 logs before filtration (*15*). An equally satisfactory titer for adenovirus 4 vaccine has been reported by Bierly (*17*) and for rubella vaccine by Plotkin (*18*).

GENETIC STABILITY OF VIRUSES PROPAGATED IN HUMAN DIPLOID CELLS

We have tested the genetic stability of all three types of poliovirus prepared in MKTC and HDC using the LTE marker test and neurovirulence test (*12*, *13*). All our results proved satisfactory. The testing of vaccines prepared in HDC after the first human passage confirmed their stability. The results are shown in table 1. The seed virus for the preparation of polio vaccine was submitted to three plaque passages and several passages in human diploid cells. This apparently had no influence on the attenuation and the genetic stability of the virus.

TABLE 1.—LTE marker test 7–10 days after first human passage

Virus types (%)	Koprowski MKTC*			Koprowski HDC†		
	1	2	3	1	2	3
Attenuated	89. 3	79. 3	72. 0	92. 7	78. 6	90. 9
Intermediate	7. 1	13. 8	12. 0	2. 4	21. 4	6. 1
Virulent	3. 6	6. 9	16. 0	4. 9	—	3. 0

*Polio vaccine prepared in monkey kidney tissue culture.
†Polio vaccine prepared in human diploid cells.

Our experiences with Edmonston-B strain show that HDC can be used as a substrate for further attenuation of measles virus. This strain, after having undergone 19 passages in HDC, including here also three plaque passages, behaved like a further attenuated strain (Edmonston B–H–Z) in field trials. In our laboratory, Leningrad-16 was passaged five times in HDC. Postvaccinal reactions caused by Edmonston B–H–Z and Leningrad-16 measles virus vaccines are shown in table 2. The side reactions and the conversion rate of antibodies are satisfactory.

TABLE 2.—Measles vaccines—postvaccinal reactions

Type of reaction	Edmonston B–H–Z*	Leningrad-16†
None	85. 4%	76. 8%
Fever	16. 6%	23. 2%
Rash	2. 6%	6. 2%
Conversion of antibodies	94. 5%	94. 3%
Number of children in follow-up	6, 063	5, 500

*Edmonston-B strain which underwent 19 further HDC passages and 3 plaque passages at the Institute of Immunology, Zagreb.
†Leningrad-16 strain which underwent 5 further HDC passages at the Institute of Immunology, Zagreb.

Table 3 shows postvaccinal reactions caused by measles vaccine (Leningrad-16) prepared in the primary cell culture of guinea pigs and in HDC in Dr. Andzhaparidze's laboratory (*15*). Once again the results obtained show that Leningrad-16 strain displayed no increased virulence after five passages in HDC and that the reactions and the conversion rate of antibodies were satisfactory.

Dr. Andzhaparidze did some other laboratory tests which also proved the stability of Leningrad-16 measles vaccine prepared in HDC. His comparative tests in newborn puppies and in Cercopithecus monkeys, with the same strain propagated in primary tissues of guinea pigs and in HDC, clearly showed that the pathogenic properties of the strain remained similar and that the HDC passage had no influence on the characteristics of the virus. Therefore, experiments on both test animals and children

TABLE 3.—Measles vaccines—postvaccinal reactions*

Type of reaction	GPKC†	HDC‡
None	73. 1%	88%
Fever	26. 9%	12%
Rash	10. 1%	4. 8%
Conversion of antibodies	83. 0%	95. 2%
Total number of children vaccinated	59	42

* Data obtained from Dr. O. G. Andzhaparidze.

† Leningrad-16 strain in guinea pig kidney cells.

‡ Leningrad-16 strain in human diploid cells.

showed that the passage in HDC does not lead to alterations in the pathogenic properties of Leningrad-16 measles virus.

The HDC substrate was also used by Plotkin for further attenuation of rubella virus and his results were satisfactory.

CONVERSION RATE AND IMMUNIZING PROPERTIES OF VACCINES PREPARED IN HUMAN DIPLOID CELLS

The examination of the conversion rate of antibodies in polio vaccines (type 1, 91%; type 2, 80%; and type 3, 90%) and measles vaccines (Edmonston B–H–Z strain, 94%; and Leningrad-16 strain, 94%) gave satisfactory results which did not significantly differ from the results obtained with these vaccines prepared in other substrates. Present information collected from our field trials confirms these laboratory findings.

According to the information supplied by Dr. Andzhaparidze (*15*), several lots of measles vaccines were prepared between 1964 and 1967, using HDC as substrate. Until 1967, 182 children were vaccinated and followed up. Antibodies against measles were determined periodically. Vaccinated children were observed from 5 months to 3 years.

The conversion of antibodies and the dynamism of formation of antibodies in this period were characteristic of a live measles vaccine and they compared well with similar preparations produced in other substrates. A group of 163 children were clinically followed up. In comparison with the control group there was no significant difference in incidence of diseases.

The results of Bierly (*17*), obtained with adenovirus type 4 vaccine, and those of Plotkin (*18*), with rubella virus vaccine, show that all the viruses concerned retain their immunizing properties after propagation in HDC.

Therefore, we can say that according to the results of both laboratory and field tests there is no significant difference in immunizing properties of vaccines prepared in HDC or in other substrates.

FIELD TRIALS

The vaccines prepared in HDC have already been given to a large number of human beings. In Yugoslavia more than 400,000 children have been orally vaccinated against poliomyelitis (*12*, *13*, *19*, *20*). In the United States over 50,000 individuals have been vaccinated by the oral route against adenovirus 4 infection (*17*, *21*, *22*). In the U.S.S.R. and Yugoslavia nearly 30,000 children have been vaccinated against measles (*15*). In the United States a small group of children have received rubella vaccine prepared in HDC (*18*).

Table 4 sums up the clinical use of vaccines prepared in HDC. If we take into consideration the actual number of vaccinated human beings, of which there is no record in the literature at the present time, it appears that over 1 million persons have been vaccinated by the oral route with vaccines prepared in HDC, and nearly 40,000 by the parenteral route.

Live, Oral Polio Vaccine

In Yugoslavia 130,170 children have been vaccinated with type 1 polio vaccine; 75,130 children with type 2; 75,250, with type 3; and 333,340 children with trivalent polio vaccine (types 1, 2, and 3). A total of 613,890 doses of polio vaccine have been used. During the 3 years after vaccination 5,313 children were followed up. Half these children received the Koprowski strain type WM–3 polio vaccine prepared in MKTC. The diseases recorded during this period were listed according to the World Health Organization classification and analyzed at the following intervals of observation: 0–3 months, 3–6 months, 6–12 months, 12–24 months, and 24–36 months. The purpose of these observations was to show if there was evidence of some differences between groups vaccinated with HDC or MKTC polio vaccines with respect to the incidence of diseases which could have a longer or a shorter incubation after vaccination. The results of the follow-up had to answer the question whether HDC as a substrate for virus vaccine production would give rise to some diseases, during the period of observation, caused by an agent present in HDC and not present in MKTC (*12*,*13*,*19*,*20*,*23*).

The groups of vaccinated children were similar. The health of the vaccinees was assessed through clinical diagnoses made by their physicians. There was no difference in the incidence and kind of diseases and symptoms recorded for the groups vaccinated with the HDC vaccine and the groups vaccinated with the MKTC vaccine.

Our findings support previous observations and laboratory evidence that HDC are free from the virus of infectious hepatitis or any other known pathogenic agent.

Table 4.—Experiences with human diploid cells in man

Vaccine	Author of report	Country	Year	Inoculation route	Number of vaccinees	Age group of vaccinees
Poliomyelitis	D. Ikić	Yugoslavia	1963–67	Oral	400, 000	Children
Adenovirus type 4	M. Z. Bierly	United States	1964–67	Oral	400, 000	Young adults
Measles	O. G. Andzhaparidze	U.S.S.R.	1964	Parenteral	163	Children
	O. G. Andzhaparidze	U.S.S.R.	1967	Parenteral	2, 707	Children
	D. Ikić	Yugoslavia	1966, 1967	Parenteral	25, 000	Children
Rubella	S. A. Plotkin	United States	1965–67	Parenteral	100	Children

Adenovirus Type 4 Vaccine

Bierly has reviewed clinical studies carried out between 1964 and 1966, and has reported that more than 42,000 persons received oral adenovirus 4 vaccine in enteric-coated tablets in that period (*17*). At least 29,000 persons from this group had not had neutralizing antibodies in measurable amounts. The administration of freeze-dried adenovirus 4 vaccine in enteric-coated tablets caused a selective infection of the gastrointestinal tract in virtually all persons not possessing antibodies. The vaccine reduced significantly the incidence of acute febrile respiratory diseases caused by adenovirus 4. Over the period 1966–67 the number of immunized subjects was increased to approximately 400,000, principally among the armed services personnel.

The use of adenovirus vaccine, live, oral, type 4, prepared by propagation of the virus in the WI–38 HDC, did not cause any abnormal sign, symptom, or other side effect in the carefully followed up groups of adults. It may be concluded that the oral administration of freeze-dried live adenovirus 4 vaccine prepared in the WI–38 cells is a simple, safe, and efficient method for the active immunization of adults against acute respiratory diseases caused by adenovirus type 4.

Measles Vaccine

Dr. Andzhaparidze (*15*) carried out a 3-year follow-up of 163 children and concluded that live vaccine against measles prepared with Leningrad-16 strain propagated in HDC shows mild postvaccinal reactions and high immunogenic properties. No pathological changes or diseases among the vaccinated children have been recorded.

On the basis of these results Dr. Andzhaparidze was granted approval to continue with the vaccination of children in 1967 and to enlarge clinical observations of live measles vaccines prepared in HDC. This time 2,707 children, 1–7 years old, with no history of measles, were vaccinated with the same lot of measles vaccine.

In the last 2 years, in several republics of Yugoslavia, more than 25,000 children have been vaccinated with live measles vaccine, prepared with Edmonston B–H–Z or Leningrad-16 measles viruses. Our experiences, reported by the clinicians taking part in this study, agree with the observations made by Dr. Andzhaparidze.

Live Rubella Vaccine

Live rubella vaccine has been prepared by Dr. Plotkin. After 21 passages the virus appeared to have the properties of attenuation. Rubella virus at the 25th passage level showed a low level of nasopharyngeal excretion (*18*) and has been used in tests in increasing numbers of children. The results of over 100 vaccinations showed a lack of reaction in vaccinees, no contagiousness, and good antibody production.

CONCLUSION

The oral administration of live polio vaccine to children, and adenovirus 4 vaccine to adults, as well as the parenteral inoculation of live measles and rubella vaccines to children, did not give rise to any pathological reactions in vaccinees which would be caused by the substrate itself during the follow-up interval. These observations and the results of laboratory tests on WI–38 HDC, which were negative, notwithstanding the long and exhaustive tests for extraneous agents, permit us to conclude that this substrate for virus vaccine production is safe on the basis of our present knowledge. The titer and the genetic stability of viruses propagated in HDC, and the high immunogenicity of the vaccines—all this argues in favor of the suitability of this system for the production of virus vaccines.

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Vaccines Produced in Diploid Cell Lines—Discussion

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THE question has often been asked whether improvements could and should be made in the production of virus vaccines to increase the safety of their use. This does not infer that virus vaccines are dangerous, but these problems of improvement have concerned the Cell Culture Committee, set up under the auspices of the Permanent Section for Microbiological Standardization, and at our fourth meeting this year we reviewed the stages of cell culture production that required greater attention.

At the outset, the Committee were agreed that, no matter how intense the surveillance of vaccinees may be, it was most unlikely that the safety of a new substrate for vaccine production could be judged by long-term follow-up in man. Only in the event of an untoward reaction occurring a relatively short time after vaccination would the cause and effect be linked. Thus the only way in which the safety of the cell culture system may be improved is to investigate each stage of the production process and, where possible, place greater emphasis on standardization.

The production of media in large quantities in the powdered form has eliminated much of the batch-to-batch variation, but the use of pretested media of this kind is not universal. Perhaps the most variable constituent of cell culture media is serum, and only recently has more attention been given to this component. By and large, the collection of the blood from which the serum was derived was in the hands of personnel unaware of the marked effect small differences in collection and handling technique might make, and it was suggested that the recording of a number of biochemical parameters, such as hemoglobin content, total protein, γ-globulin content, and specific gravity, would do much to standardize the serum. Above all, however, the growth-promoting properties of each batch should be checked before use in the production of a cell substrate.

These days we hear much of the importance of *Mycoplasma* and it is particularly important that sera, cell cultures, and vaccines are free from these contaminants. A recent investigation has shown, however, that some laboratories with a high scientific reputation have been unable to detect a low infectious dose of *Mycoplasma* in cell cultures. Autoradiographic studies with tritiated thymidine or uridine are showing great promise in the detection of these agents.

The most exacting questions discussed by the Cell Culture Committee were the steps we should be taking toward the establishment of standardized cell substrate. Although we have no evidence that vaccines produced on monkey kidney cell cultures are dangerous, almost everyone is conscious of the potential risks involved in their use. On at least one occasion a living agent survived an inactivation process and remained undetected for a time in final vaccine. Recently a disease was transmitted from monkeys to man via the blood and organs and survived to the cell culture stage. How long will it be before a simian virus pathogenic for man will remain undetected in final vaccine, causing a tragic accident? Our present knowledge of virology is such that this possibility is not out of the question when monkey kidneys are used, and we must not let this happen.

The use of chick embryos or their cell cultures is a major advance, but in spite of the fact that yellow fever vaccine, known to be heavily contaminated with avian leukosis viruses, appears to be safe, we have limited the production of measles vaccine to cell cultures free from avian leukosis. A tissue derived from animals grown under controlled conditions is far superior to tissue of monkeys caught in the wild, but have we gone far enough, and is this the ideal? As far as I am aware, no animal or bird is free from viruses and it would be a greater advantage to use a tissue frozen until such time as exhaustive tests had freed it from contaminants. The "cell seed system" is as important to the standardization of vaccine production as our "virus seed system" and is regarded as a fundamental necessity in manufacture today. Without being specific about any animal species, some principles for the selection of a suitable tissue can be followed:

1) The tissue should be derived from normal tissue and remain normal throughout its propagation. Fetal tissue is to be preferred because it will have had the least chance of being contaminated.
2) It should be frozen and not used until exhaustively tested for contaminants.
3) Ideally, it should be able to grow the majority of human viruses so that it is suitable for the production of the majority of human virus vaccines.

The Cell Culture Committee have been anxious to learn of suitable candidate cell strains, but so far only WI-38 appears to satisfy these criteria of acceptance. There are those who object to the use of these cells on theoretical grounds, but in general there are two charges against them: (*a*) They are human cells and thus may carry a human oncogenic agent;

(*b*) they may have a slow virus that may only become apparent years after the inoculation of human subjects.

The evidence to date is that primate oncogenic viruses do not give rise to tumors in the species in which they are indigenous, but have always expressed their oncogenicity in a foreign species. As far as slow viruses are concerned, these are known to cross species barriers and, as with other animal viruses, animal tests must be used for their detection rather than long-term observations of vaccinees. On both these counts, therefore, animal tissue would not be safer than human tissue for production of human virus vaccine.

The WI–38 cell line has been tested for the presence of extraneous agents in more laboratories than any other cell culture, and none has been found. If it is still believed that an undetectable virus is present in these cells, such a virus must: (*a*) have passed the placental barrier; (*b*) be able to remain latent in culture and produce no apparent karyologic, cultural, or morphologic changes during many serial passages; (*c*) have been able to produce no changes in a wide variety of other cell types, both human and animal; (*d*) have produced no observable effects in any species of experimental animal; and finally, (*e*) be able to propagate in culture at least to the extent that it is not lost by dilution for 20–30 cell generations.

Taken in conjunction, this highly unlikely combination of circumstances must be weighed against the known difficulties inherent in tissue such as monkey kidney cultures.

Many vaccines have been made with WI–38 cells as the substrate. These vaccines have been given both orally and parenterally to man with surveillance for 2–3 years.

So far, no vaccine prepared on WI–38 cells has been licensed for sale; it is inevitable that the acceptance by committees of such a fundamentally different approach to vaccine production will take time. This is as true for the United Kingdom as for the United States of America. However, it is an encouraging sign to see so much work in progress in the use of these cells; we must move towards this ideal situation and establish a "cell seed system" for our future vaccines.

If there are any objections to this, the Cell Culture Committee would be glad to know of them so that we may investigate the establishment of new cell substrates free from such objections.

GENERAL DISCUSSION

Dr. H. Tint (Wyeth Laboratories, Inc.) : I think it is appropriate, at this point in the Conference which deals with the mass use of live vaccines prepared on diploid cells, to refer again to some statistics mentioned earlier, that perhaps might correct Dr. Ikić's report a little but would not minimize the significance and importance of his report.

The reference to the report of Dr. Bierly, summarizing clinical experience with adenovirus type 4 vaccine propagated on WI–38 cells, referred in fact to a report which summarized investigations carried out by many other individuals in various laboratories, principally among personnel of the armed services, which through 1966 did in fact aggregate to something of the order of 40,000 immunized subjects.

Subsequently, in the respiratory disease season of 1966 and 1967, the number was in fact increased considerably again by these investigators, some of whom are present in this room, to a magnitude approaching a half million immunized subjects. I believe the provisional planning at the moment is to double this number to something of the order of 1 million subjects by the completion of the forthcoming respiratory disease season.

About the efficacy of this vaccine I need not say much. Many reports attest to its efficacy, in terms of the conversion of antibody negatives, of the order of magnitude of 90, to 95, to 100% and also in terms of protection against epidemic disease, in reducing specific disease and associated hospitalization and admissions.

As to the safety of this form of vaccination with material produced on this particular substrate, the situation is approaching academic proportions because a consensus has already been reached by the people responsible for the planning, conduct, and authorization of these trials that, in fact, for the purpose intended, it was indeed a safe procedure to undertake. And the subject numbers are, in fact, only two to three magnitudes away from matching the entire population of the United States.

Dr. E. L. Buescher: I would like to amplify on Dr. Tint's comments perhaps to give you the first accurate indication of the number of Americans who have been fed the WI–38-propagated adenovirus type 4. The number probably is of the order of 250,000 to 300,000. I still have about 200,000 doses in my freezer.

There is a reason for this. It is perfectly safe to administer this vaccine to individual men. But in the process of its mass application from the middle of November of 1966 through the end of April 1967, we have very effectively created an ecological vacuum in the military populations that received the vaccine. This ecological vacuum has been like all ecological vacuums—nature abhors them. It has been filled by yet other pathogens, so that at this time in the United States, the significant pathogen causing acute respiratory disease in the young, basic-combat trainees is no longer type 4 but adenovirus type 7. Furthermore, on at least one basic-combat training post, adenovirus type 21 has also been established.

From a biological standpoint, it does not make sense to extend the use of adenovirus type 4 to prevent disease caused by other types. Caution, therefore, is going to be exercised in the administration of vaccine to basic-combat trainees at least as far as the Army is concerned because, again, as I will have a little bit more to say later, the risks have to be balanced against the needs. Right now, in a circumstance where respiratory disease is not associated with adenovirus type 4, it does not seem logical to immunize with that preparation.

Dr. J. Mills (National Institute of Allergy and Infectious Diseases) : In view of Dr. Miller's and Dr. Robbins' comments on the use of epidemiology in studying vaccines given to the population, I wonder if Dr. Perkins would further justify the statement of his committee that this method was not useful in evaluating tissue culture substrates for vaccines. Perhaps Dr. Miller or Dr. Robbins would comment on this also.

Dr. F. T. Perkins: Perhaps my statement needs a little qualification, since we hear so much about the need for following the vaccinees when a new substrate is used and the questions always arise: How many should have the vaccines and for how long should they be observed?

In the Committee we were balancing the dangers that we know exist with the use of monkey kidney and the risks we shall continue to run by using this cell substrate against holding up the use of a new cell substrate such as WI-38 or any other stabilized cell substrate that has been frozen and examined for freedom from extraneous agents. And we didn't think that in a finite time of, let us say 5 years or 10 years, we were likely to get an answer. Having done this we would have held up the use of the new standardized cell substrate for no purpose.

I have been very impressed with Dr. Miller's schemes for observing the vaccinees and the point was made earlier by Dr. Robbins that this observation should start immediately when a new vaccine is used. I think this is vitally important and I am very glad to see that it is being done here. But I do not think that this should be used as an excuse for holding up a new standardized cell substrate.

Dr. F. C. Robbins: I think Dr. Miller has left the room, so I will have to respond to this. I was going to take mild exception to Dr. Perkins' statement, but now that he has clarified it I don't take exception. Because I think that the points he makes about making a reasonable judgment on the basis of reasonable data are inescapable. It is not possible to wait long enough to be absolutely sure. We will never be that sure.

On the other hand, I would not be so pessimistic as to feel that epidemiologic investigations would not be capable of yielding information of value and that, therefore, they should not be attempted. I would view this as a continuing type of surveillance rather than just an evaluation of a particular product before releasing it for use.

We are now faced with the need to have an almost automatic continuing kind of surveillance, because the time factors involved are unknown and are quite clearly long. And here I am not just talking about cancer; I am talking about other possibilities. One can frighten oneself to death if one wants to. We would be absolutely paralyzed.

I don't quite adopt the bold and vigorous point of view that Dr. Hilleman adopts, but I also don't think we can afford to be so cautious that we don't do anything. However, one does have to balance, not just known risk, but potential risk against what one thinks the value of the product or procedure might be.

Dr. B. A. Rubin: I would like to say something about Colonel Buescher's comment. A most important concern of this meeting is the question of the use of oncogenic agents in vaccines, including those agents which may be called oncogenic on the basis of laboratory effects. Earlier, Dr. Huebner called several agents oncogenic which had never produced a tumor in any species of animal but had produced changes in tissue culture. We have strains of adenovirus type 7 suitable for vaccine use that have produced tumors in fewer than 1% of inoculated hamsters after more than a year. How important is it to be concerned about hypothetical problems in the face of real problems?

The number of adenoviruses that are epidemic or have epidemic capabilities are fairly limited, but these include the "mildly oncogenic" types. It is relatively simple to prepare vaccines from these agents that would be very similar to the very effective adenovirus type 4 vaccine. The stigma cast by questionable levels of oncogenicity needs to be considered more realistically if such vaccines are to become available.

If you follow the logic of Colonel Buescher's suggestion about the type 4 vaccine, you should stop measles vaccination, smallpox vaccination, and so on, because you don't happen to see the disease around at the moment. I think you will find if you stop adenovirus type 4 immunization, you would very soon see the return of this disease.

Dr. Buescher: It hasn't.

Dr. H. Koprowski: Could I intervene now? This discussion of an enormously important subject—namely, of the question of eradication—is slightly beyond the scope of this Conference; a whole conference could be devoted to this problem.

Since we have now only 1 or 2 minutes more, and this will be the only time when the subject of diploid cells as substrates for vaccine production can be discussed, perhaps you should limit your discussion to this problem.

Dr. Francis is laughing because I am writing the book now, he says.

Dr. P. A. Long: (U.S. Department of Agriculture Poultry Research Laboratory, East Lansing, Mich.) : Dr. Perkins made a statement that measles vaccine would be produced only in avian leukosis-free eggs or cell cultures, and the Cell Culture Committee Report says:

"Thus, although chickens have been bred under controlled conditions which are free of fowl leukosis viruses, a new infectious agent, that of Marek's disease, has been recently isolated, and only a few commercial flocks have free immunization."

What flock is completely free of all avian leukosis viruses? Also, which are free of Marek's disease? I am referring to all three known subgroups and unknown subgroups—preferably the one Dr. Vogt just mentioned to me, the possibility of a B group.

Dr. Perkins: I can't answer the question of which flocks are free from all these agents, but you mustn't take this comment out of context. The point is that this is an improvement over the use of monkey kidney tissue taken from animals caught in the jungle without there being any control over their habitat or breeding or much testing for freedom from infectious agents in the animals. This was the only point that was being made there.

Dr. R. Murray: Dr. Ikić, in order to make your presentation perhaps more comprehensive, I think it would be of value if you could inform us of the kind of follow-up that the children had who received the material which was injected.

Dr. D. Ikić: All children included in the study were under the control of their medical centers. Each child had his file in the medical center it belonged to, and the medical centers from time to time organized routine controls and examinations of children of their areas. Histories of diseases were kept in the records of the medical centers.

Immunization of Experimental Animals With Vaccines Produced in Known Oncogenic Cell Lines

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THE objections raised to the use of continuous cell lines for the production of virus vaccines are hypothetical, since no definite problem has been demonstrated. These objections for the most part relate to the potential neoplastic properties of such cell lines and to the fear that such a potential might be transmitted to man through a vaccine prepared in such a cell line.

Our investigation of these problems, if such problems do exist, has taken two courses. One, referred to earlier in the program, involved as thorough a characterization as possible of a cell line that was of interest to us as a possible substrate for virus production. The other approach was an attempt to determine, through use of model systems, whether any real problem could be demonstrated. This report deals with studies of the latter type.

As a model system representing the worst possible situation, we selected a cell line isolated from a well-known transplantable tumor and carried continuously in our laboratory for over 13½ years. This line, LLC–WRC$_{256}$, was established from Walker rat carcinosarcoma 256, and it has retained its ability to produce tumors and to kill newborn or very young rats. It is susceptible to vaccinia and the herpesviruses. This line and a C3H mouse line, NCTC 1742, established by Sanford (*1*), and which became tumorigenic *in vitro*, were included. The NCTC 1742 cells were susceptible to Coxsackie B-5 and herpes simplex viruses.

We attempted to answer the following questions:

1) Would the cell lines at the passage levels used produce tumors in their respective hosts?
2) Would animals inoculated with live virus produced in these cell lines develop tumors after inoculation?

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[1] The histopathological examinations were performed by Dr. C. G. Culbertson and the electron micrographs were prepared by Mr. T. E. Newlin. Technical assistance was provided by Mrs. L. A. Dye, Mr. J. H. Bodenmiller, and Mr. G. Weaver.

3) Would the cells after treatment with formaldehyde in the same concentration and for the same period as used in vaccine production produce tumors?
4) Would the cells treated with only the preservative used in the vaccine still produce tumors?
5) Would finished vaccine produced from virus grown in the cell lines, and which was antigenically potent, produce tumors in susceptible hosts?

The answer to question 1 was "Yes"; the answer to all the others was "No," or, at least, probably "No."

In studies with the LLC–WRC$_{256}$ line, pseudorabies virus was used. The findings from one representative experiment were as follows:

The 297th serial passage of the cell line was used. Thirty-two, 1- to 2-day-old suckling white rats were each inoculated subcutaneously with approximately 10^6 viable cells in the interscapular fat pad area. Two animals were missing on the 5th day after inoculation, but the 30 remaining rats all developed tumors by the 21st day.

A similar cell suspension was treated with 1:4000 formaldehyde for 13 days at 37°C. Each of 15, one- to two-day-old rats was inoculated with these cells. The animals were observed for 160½ weeks, until the last member of the group died. The first death occurred at 53 weeks, and there was no tumor or other specific pathology noted in this animal. Six of the fifteen animals developed tumors. These findings are summarized in table 1. Five of the six tumors were fibroadenomas of the breast and were probably unrelated to the inoculated LLC–WRC$_{256}$ cells. Rat #7 had a growth or nodule in the left axilla during the 110th week post inoculation and was sacrificed. Unfortunately, the histopathology either was not done or the report was lost.

TABLE 1.—Tumor incidence in rats inoculated with formaldehyde-treated LLC–WRC$_{256}$ cells

Rat No.	Time (wks)	Histopathological description
3	79	Benign fibroadenoma of breast
5	95	" " " "
10	121	" " " "
11	121	" " " "
14	146	" " " "
7	110	? nodule left axilla

The other 9 animals, not showing tumors, died between the 53d and 160th week. These are recorded in table 2. Only animal #2, with hind-leg paralysis, showed any clinical evidence of illness, but by histopathological examination only a mild pneumonia was noted. None of the other animals showed any gross pathology and therefore were not examined further.

A sample of the cell suspension was treated also with a 1:20,000 final dilution of Merthiolate, the preservative used in most vaccines. After the cell suspension was stored for 19 days at 4°C, the rats were inoculated and observed until the last one died at 155 weeks post inoculation. Only 2 animals developed tumors, which were noted in the 98th and 132d weeks. Histopathologically, the tumors were classified as fibroadenomas of the breast. The death records of the other 19 animals are given in table 3. No tumors were observed in any of these animals autopsied, nor was there other gross evidence of pathology suggesting the cause of death.

A 500 ml pool of pseudorabies virus was harvested from ten 16-ounce bottle cultures of 297th passage of LLC–WRC$_{256}$ cells. The raw virus pool had a titer of $10^{-7.0}$/0.5 ml when assayed in LLC–RK$_1$ cells (*2*). After Selas XFF and Selas 10 filtration, the pool titer was $10^{-6.0}$/0.5 ml. Two-tenths ml of the postfiltration virus pool was inoculated subcutaneously into the fat pad area of each of 16 newborn rats. Within 3 days all inoculated animals were dead, due to pseudorabies virus infection; thus we were unable to determine whether live virus produced in this cell line possessed any malignant properties for the newborn white rat.

TABLE 2.—Death records of rats inoculated with formaldehyde-treated LLC–WRC 256 cells but which did not develop tumors

Rat No.	Time (wks)	Observations
1	53	No gross pathology
4	89	" " "
6	104	" " "
12	142	" " "
13	143	" " "
15	160½	" " "
2	54	Hind-leg paralysis, mild pneumonia
8	119	Died, eaten
9	120	Died, badly decomposed

TABLE 3.—Death records of rats inoculated with Merthiolate-treated LLC–WRC$_{256}$ cells

Rat No.	Time (wks)	Observations
1–5	<1	Died shortly after injection
6	52	Died, no gross pathology
7	53	" " " "
8	59	" " " "
13	114	" " " "
14	118	" " " "
15	121	" " " "
16	124	" " " "
17	127	" " " "
19	133	" " " "
20	138	" " " "
21	155	" " " "
9	60	Ill, sacrificed, no gross pathology
10	89	Died, badly decomposed
12	106	" " "

The remaining portion of the virus pool was processed into vaccine by inactivation with formaldehyde. When completed, the formaldehyde was neutralized by addition of sodium bisulfite. One hundred ml was tested for inactivation by the inoculation of 25 ml into each of four 16-ounce bottle cultures of strain LLC–RK$_1$ cells. The cultures were observed for 28 days with fluid changes and subcultures performed at 7-day intervals. No evidence of residual live pseudorabies virus was detected. Since we did not want to bleed the baby rats on oncogenicity test, potency was evaluated in guinea pigs. Antibody response to pseudorabies ranged from 1:8–1:128.

Seventy-eight newborn rats were each inoculated with 0.2 ml of the finished vaccine in the same manner as previously described. The group was observed for tumor development for 154 weeks, at which time the last one died. Sixteen of 78, or about 20%, developed tumors or growths of some type during their lifespan. The nature and distribution of these tumors are seen in table 4. Of these, 10, or possibly 11, were breast tumors, most of which were benign or of a very low-grade malignancy. Otherwise, there were 3 fibrosarcomas and 1 malignant lymphoma with extensive metastases. No tumors were seen during the first year. No carcinomas or carcinosarcomas resembling the Walker rat tumor were observed.

TABLE 4.—Types and numbers of tumors observed in pseudorabies-vaccinated rats

Tumor type	Number of rats	Time sacrificed (wks)
Benign fibroadenoma of breast	5	58, 95, 101, 108, 141
Fibroadenoma of breast with metastasis	1	101
? Malignant breast tumor	2	71, 101
Fibrosarcoma of breast	1	71
Angiofibroma of breast	2	125, 125
Cystadenoma (breast or sweat gland)	1	115
Fibrosarcomas	3	89, 95, 122
Lymphosarcoma and lymphoma	1	131

Based on reports in the literature, the incidence of breast tumors in rats observed in our study is not unusual. Thompson *et al.* (*3*) reported an incidence as high as 41.6% and found the average lifespan to be about 120 weeks. Other authors (*4–8*) reported incidences ranging from 1.53–57% for different strains and/or colonies.

The remaining 62 animals, showing no evidence of tumor development, are accounted for in table 5. The survival rate of these animals was good, as only 11 were lost during the first year; slightly over 50% were still surviving at the end of the second year, even though 9 were sacrificed for histopathology during this period. All animals that died, or showed clinical symptoms and were sacrificed, were autopsied and observed for gross pathology. Histopathological examination was performed only on those animals showing some gross pathology.

TABLE 5.—Death records of rats immunized with pseudorabies vaccine but which did not develop tumors

Observations	Number of rats	Time (wks)
Died, no gross pathology	47	*
Died, eaten	1	4
Died, badly decomposed	4	45, 106, 111, 118
Died, diarrhea	3	44, 46, 79
Died, acute bacterial infection	2	15, 55
Ill, sacrificed, nephritis	1	112
Missing from cage	4	14, 22, 23, 135

*<52 weeks: 3 rats; >52, <104 weeks: 12 rats; >104 weeks: 32 rats.

Similar studies were undertaken with the C3H mouse cell line, NCTC 1742. Coxsackie B–5 virus grew to good titer in this line, and we intended to use this virus and process it into vaccine by the same method used in the production of poliomyelitis vaccine. Highly antigenic preparations were made, but none passed the safety test because traces of residual live virus were detected. This was dropped, therefore, in preference to herpes simplex virus, which also grew to good titer in strain NCTC 1742.

The findings of these studies will be presented as a compilation of data from numerous experiments. In assessing the transplantability of the strain for inbred weanling C3H mice, we inoculated 143 animals intramuscularly with approximately 10^6 viable cells. Fifty-five, or approximately 38%, developed tumors, while 88 survived without evidence of tumors for 42 weeks, when the observations were discontinued. The tumors produced by this cell were rapidly growing intramuscular sarcomas, apparently of connective tissue origin, but which had invaded the muscle tissue. This line did not produce tumors with the same degree of regularity or in as high incidence as the LLC–WRC_{256} line of cells.

Herpes simplex virus titers before filtration or inactivation ranged from 10^{-5}–$10^{-7.0}$/0.5 ml. One group of 25 weanling C3H mice was inoculated intramuscularly with 5.75 logs of postfiltration live virus. No tumors developed during 42 weeks of observation and 21 animals survived the 42 weeks.

Herpes simplex vaccines were prepared from virus grown in NCTC 1742 cells. After Selas XFF and 10 filtration, the virus was inactivated with formaldehyde at a final concentration of 1–1,000 for 48 hours at 37°C. After neutralization with sodium bisulfite and dialysis, 100 ml was safety tested in LLC–RK_1 cells. All lots passed this test. Potency of the vaccine was measured by mouse-protection tests and guinea-pig antibody response. Forty percent of the immunized mice challenged by intracerebral inoculation of approximately 150 MLD50 survived. Guinea pigs developed titers of 1 : 32–1 : 64.

VIRUS VACCINE PRODUCTION

One hundred and twenty-three weanling C3H mice were vaccinated intramuscularly. One hundred and fourteen survived for 42 weeks without evidence of tumor development or other neoplastic disease.

Why some cells cultured *in vitro* undergo malignant transformation is not completely understood, though it is known that some chemical carcinogens and viruses can produce such changes. In the NCTC 1742 cells, possibly the development of tumorigenicity was the result of a latent virus infection, since Hall *et al.* (*9*) have reported the presence of virus-like or C particles in these cells. This we confirmed in our laboratory by electron microscopic examination. A representative thin section of a cell containing such particles is seen in figure 1. Whether this particle is responsible for the tumorigenicity of strain NCTC 1742 is not known, but there are other malignant cell lines in which virus-like particles have not been detected; thus their tumorigenic properties may be due to other factors. Strain LLC–WRC_{256} falls into the latter category. Many electron microscopic fields of thin sections of LLC–WRC_{256} cells were examined without detection of virus-like particles. In addition, fluids harvested from LLC–WRC_{256} cells were inoculated into ten different cell culture systems and carried through four blind passages in an attempt to detect any virus present. Three human cell lines, HeLa, AV_2, and LLC–He_1, were included, along with primary rhesus and green monkey kidney, three continuous cell strains derived from rhesus monkey kidney, one from green monkey kidney, and one from rabbit kidney. No cytopathic or hemadsorbing agents were detected. Viable LLC–WRC_{256} cells were also inoculated into Swiss white mice without evidence of tumor production or infectious disease. Thus the two model systems included both virus-containing and, presumably, virus-free tumorigenic cell strains.

Within the limits of these model systems there appeared to be no definite contraindication to the immunization of experimental animals with filtered, inactivated virus vaccines produced in cell lines tumorigenic to the host involved. In view of these findings, it would seem that immunization with vaccines made in heteroploid cell lines, derived from normal tissue and not demonstrated to be tumorigenic, would be of even less concern.

These studies, of course, dealt with formaldehyde-inactivated vaccines and with viruses producing a cytopathic effect in the cell strains under study. Since the use of live, attenuated vaccines is now more in vogue, similar studies should be made to include such products. It was demonstrated, however, that the growth in, and destruction of, NCTC 1742 cells by herpes simplex virus, plus a clarifying filtration, removed the tumorigenic potential of these cells. Some viruses used as live, attenuated vaccines produce CPE, while others do not. Rubella is an example of the latter type. We plan to undertake studies similar to those just described with live rubella virus vaccine grown in the BHK_{21} cell line, which, purportedly, is transplantable to hamsters.

Despite the preliminary nature of these studies, inactivated and antigenically potent vaccines produced in demonstrated tumorigenic cell lines produced no clear-cut evidence of neoplasia when used in the immunization of the respective tumor-susceptible hosts. These data are not extensive enough to make any broad or general conclusions, since they were essentially negative. A positive result would possibly have added to the fear of some investigators to use continuously propagated cell lines for vaccine production. In the final analysis, each candidate cell line and each virus under consideration for vaccine production will have to be examined and considered as individual entities in respect to safety.

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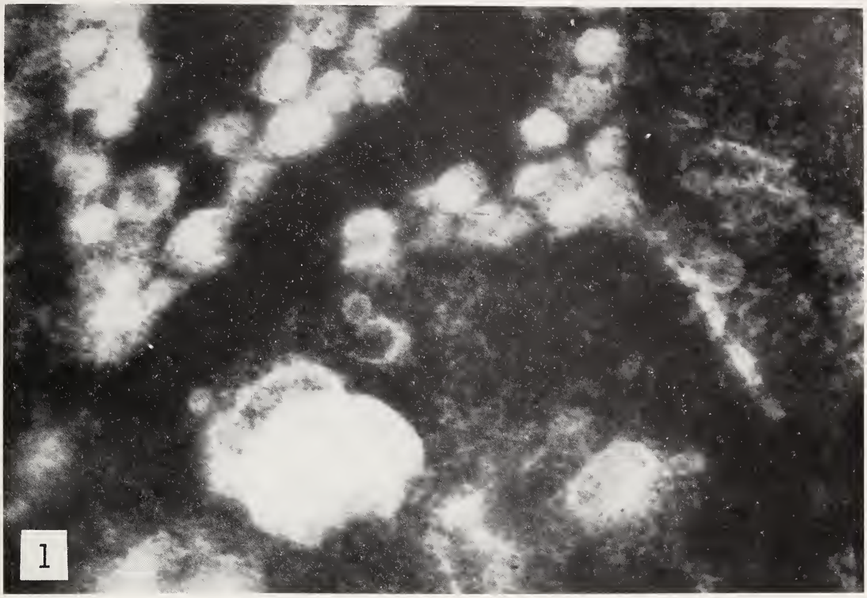

FIGURE 1.—Electron micrograph of thin section of an NCTC 1742 cell showing 100 mμ virus-like particles. Fixed with 0.6% $KMnO_4$ and stained with lead acetate. Original plate approximately × 57,000.

GENERAL DISCUSSION

Dr. T. Francis, Jr.: May I ask Dr. Hull what strength formalin was used to treat the cells of the original cultures which he said were inactivated or that at least after formalin treatment there was no tumor production.

Dr. R. N. Hull: The particular experiment which I reported this morning was done according to the poliomyelitis procedure by using 1 : 4000 formalin at 36° for 13 days.

Dr. L. Hayflick: I would agree largely with what Dr. Hull has said. If our choice was only between primary tissue and these heteroploid populations, it then seems clear to me that the points that Dr. Hull made are quite valid, especially if one assumes that one would be using a heteroploid population derived from normal tissue. However, that is not the only choice. The choice, as we all well know, should be between a heteroploid population and a diploid population derived from normal tissues.

The most reasonable conclusion would be that, given this choice, one would much prefer to produce vaccines in those cells that adhered more closely to what we like to think of as a normal cell population. We would also take comfort in the fact that, if the normal diploid populations did acquire abnormal properties that escaped our detection, this would not be catastrophic since Dr. Hull's results imply that cells having apparently malignant properties are possibly safe for vaccine production.

I would, nevertheless, like to know what specific advantages there would be in using a heteroploid population derived from normal tissue over a normal human diploid cell strain in which the cell yield is virtually limitless.

Dr. R. N. Hull: The easiest comment, I believe, to make is to answer the last question raised by Dr. Hayflick. I think it should be obvious from the technical standpoint that it would be easier to handle a cell that grows indefinitely and can be subcultured at a 1 : 10 split ratio or greater.

Incidentally, strain LLC–MK_2 cannot be grown in fluid culture. According to Dr. Hayflick, this is another marker of a normal cell.

Cells which could be grown in fluid suspension, of course, would add further technical advantage to production procedures. I think this is the principal advantage.

The other comment I would like to make is that I don't see why there has to be a single choice, and this has been brought out earlier by several of the speakers. We should have more than one cell or cell type so that we can compare something to something, as I believe, Dr. Robbins stated.

Dr. J. Furth: With respect to the rat, the findings of Dr. Hull seem conclusive. The tumors observed are indigenous and common in rats. There is no evidence that rats carry oncogenic viruses.

With respect to mice, I am missing a statement on viruses carried in the strains used, notably leukosis, polyoma, and mammary tumor viruses.

Such an experiment would be more informative, if done in a mouse strain which is "certified" free from such viruses and if the results were compared with the results in mice which carry known oncogenic viruses. Supplementation of the tumorigenic potentiality with karyologic findings would also be of interest.

Veterinary Vaccines

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VETERINARY biologics produced under U.S. Veterinary Licenses issued by the U.S. Department of Agriculture approximated 8,300,000,000 doses in the fiscal year ending June 30, 1966. That is counting separately each fraction of products containing two or more viruses.

Cell-culture origin virus vaccines approximated 177,000,000 doses, slightly exceeding 2% of the total production. Table 1 shows the production of cell culture virus vaccines by animal species.

Tables 2 through 7 show cell culture virus vaccines for use in cattle, dogs, cats, horses, mink, rabbits, sheep, swine, and poultry; the year in which each vaccine was first licensed; the number of doses for fiscal year 1966; and the virus taxonomy and cells used for production.

A complete list of licenses and veterinary biological products licensed is available on request from Veterinary Biologics Division, ARS, U.S. Department of Agriculture. Yearly production figures are published in Veterinary Biologics Division Notices, also available on request. Outlines of production methods required for licensing veterinary biologics are considered the property of the applicant. Details concerning these outlines

Table 1.—Cell culture virus vaccines by species

Species	Doses	Species	Doses
Cattle	32, 500, 000	Sheep	NA
Dogs	13, 200, 000	Swine	29, 700, 000
Cats	140, 000	Poultry	96, 250, 000
Mink	3, 100, 000	Others	1, 320, 000
Horses	590, 000		
Rabbits	NA*	Total	176, 800, 000

*Not available.

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[1] U.S. Department of Agriculture.

TABLE 2.—Cell culture virus vaccines for use in cattle

Species and vaccines	Doses
Cattle: Total doses	32, 500, 000
1 Bovine rhinotracheitis MLV 1960 Miscellaneous herpesvirus.	25, 700, 000
2 Bovine virus diarrhea MLV 1963 Possible myxovirus.	6, 800, 000
3 Parainfluenza-3: KV 1967–MLV 1966	NA*
1, 2, 3—Bovine kidney: Primary cells. Cell lines. Continuous cell line.	
1—Canine kidney: Primary cells.	
3—Rabbit kidney: Primary cells.	
2—Porcine kidney: Primary cells.	

*Not available.

TABLE 3.—Cell culture virus vaccines for use in dogs

Species and vaccines	Doses
Dogs: Total doses	13, 200, 000
1 Canine distemper 1960, myxovirus	6, 450, 000
2 Measles vaccine 1965, myxovirus.	
3 Canine hepatitis 1957, adenovirus	5, 700, 000
4 Rabies vaccine 1964, myxovirus	1, 050, 000
1—Chicken-embryo primary cells. 3—Calf kidney primary cells. 1, 2, 3—Dog kidney primary cells. 1, 3—Ferret kidney primary cells. 4—Hamster kidney primary cells. 3, 4—Pig kidney primary cells. 1, 3—Dog kidney diploid cell line. 1, 3, 4—Dog kidney heteroploid cell line.	

TABLE 4.—Cell culture virus vaccines for use in cats and minks

Species and vaccines	Doses
Cats: Total doses	140, 000
Feline distemper KV 1965. Unclassified panleukopenia virus. Cat kidney: primary cells. epithelial cell line.	
Mink: Total doses	3, 100, 000
Distemper vaccine (mink) LVM 1962. Myxovirus. Chicken embryo—primary cells.	

TABLE 5.—Cell culture virus vaccines for use in horses, rabbits, and sheep

Species and vaccines	Doses
Horses: Total doses	590, 000
Encephalomyelitis eastern and western KV 1965. Arboviruses, Group A. Chicken embryo—primary cells.	
Rabbits: Total doses	NA*
Myxomatosis LVM 1965 pox-tumor virus. Rabbit kidney—primary cells.	
Sheep: total doses	NA
Bluetongue LVM 1954 ungrouped arbovirus. Bovine fetal kidney—primary cells.	

*Not available.

TABLE 6.—Cell culture virus vaccines for use in swine

Species and vaccines	Doses
Swine: Total doses	29, 700, 000
Hog cholera vaccine LVM 1952. Bovine virus diarrhea. Mucosal disease. Hog cholera virus complex. Possible myxovirus. Porcine kidney primary cells. Porcine kidney PK2-A established cell line. Porcine bone marrow—primary cells. Porcine bone marrow-lungs-pancreas—primary cells. Rabbit kidney—primary cells. Infected swine buffy coat—established cell line.	

TABLE 7.—Cell culture virus vaccines for use in poultry

Species and vaccines	Doses (fiscal year 1966)
Poultry: Total doses	96, 250, 000
Laryngotracheitis LV 1963	71, 360, 000
Herpesvirus, subgroup A. Chicken embryo—primary cells.	
Fowl pox LV 1965	20, 000, 000
Avian poxvirus. Chicken embryo—primary cells.	
Newcastle disease LVM 1961	4, 890, 000
Paramyxovirus. Porcine kidney—primary cells.	

may be obtained only from the owners. The Virus-Serum-Toxin Act (March 4, 1913, 37 Stat. 832–833, 21 U.S.C. 151–158) prohibits worthless, contaminated, dangerous, or harmful products. Licensing requirements include production methods and support data, with laboratory and field tests for purity, safety, and potency.

Table 8 shows viruses used in cell culture vaccines and the fiscal year 1966 production.

Viruses used must be listed and defined as to source strain, proportions, identification, purification, adaptation, modification, and maintenance. Biochemical properties, growth, titers, virulence or avirulence, antigenicity, and immunogenicity must be established for the range of subcultures to be used in production. Each seed pool must be tested for identity, immunogenicity, purity, and safety. Only seed virus free of bacterial, fungal, mycoplasmal, and viral contamination may be used for production.

Table 8.—Viruses in cell culture vaccines and fiscal year 1966 production

Cell culture vaccine viruses		Doses (fiscal year 1966)
Adenoviruses:	Canine hepatitis	5, 700, 000
Arboviruses:	Eastern viral encephalitis	590, 000
	Western viral encephalitis	
	Bluetongue	NA*
Herpesviruses:	Bovine rhinotracheitis	25, 700, 000
	Infectious laryngotracheitis	71, 360, 000
Myxoviruses:	Bovine virus diarrhea	6, 800, 000
	Parainfluenza-3	NA*
	Canine distemper	6, 450, 000
	Measles	NA*
	Rabies	1, 050, 000
	Hog cholera	29, 700, 000
	Newcastle disease	4, 890, 000
Poxviruses:	Fowl pox	20, 000, 000
	Myxomatosis	NA*

*Not available

Primary cell cultures must be derived from normal tissues and animals and must maintain a normal control appearance through the virus growth period and harvest.

Special tests are required for indigenous or adventitious agents such as *Mycoplasma gallisepticum*, *Brucella abortus*, bovine virus diarrhea virus, and lymphocytic choriomeningitis virus.

Cell lines and cell strains may be used for vaccine production provided they are derived from normal tissue, stable and free of indigenous or adventitious agents and oncogenicity.

Cell characterization includes:

1) History, subculture number or passage, growth characteristics, plating efficiency, morphology, and karyotype with chromosome numbers and frequency.
2) Sterility tests for mycoplasma, bacteria, fungi, and viruses.
3) Tumorigenicity tests, species of origin, and antigenic identification.
4) Virus susceptibility and stability.

Cell history includes:

1) Determination that the tissue of origin was normal and not neoplastic.
2) Statement as to whether the tissue was adult or embryonic.
3) Animal species.
4) Organ of origin.
5) Isolation date, procedure, cell type, and description of primary culture.
6) Line or strain designation.
7) Cloning or selection by other methods.
8) Maintenance.

Tumorigenicity may be determined by inoculation of conditioned laboratory animals with unlysed cells without and/or with vaccine virus. Cheek pouch inoculation of cortisone-conditioned hamsters and inoculation of mice conditioned by irradiation have been found satisfactory.

Susceptibility to vaccine viruses, quantity, and antigenic quality produced should be established for the range of cell and virus subcultures to be used in production. Consistent susceptibility to all the viruses which a cell line will support through a range of passages is a good criteria of stability and purity. Study of the susceptibility of a cell line to selected viruses may unmask associated, helper, or synergistic viruses which could otherwise remain undetected.

Culture of lysed cells and fluids in available cell systems and inoculations in various animal species may also be useful to detect indigenous and adventitious agents. Fluorescent antibodies for viruses, tumor antigens, and cells are recommended tools for detection of indigenous or adventitious agents and cross contaminants of cell lines and strains. Electron microscopy may detect unknown agents for which no other method of detection has as yet been devised.

All studies should be repeated periodically to check the stability of cells and viruses and the reproducibility and reliability of tests, and also to take advantage of newer techniques and available knowledge.

In evaluating cell cultures for virus vaccine production, it is worthwhile to consider available data about virus tissue vaccines prepared from infected animal tissues or organs, such as brain, heart, lung, liver, spleen, kidney, gut, bladder, skin warts, amino-allantoic fluid, chorioallantoic membrane, yolk sac, and whole chicken embryo.

VIRUS VACCINE PRODUCTION

Table 9 lists the most important tissue virus vaccines and the number of doses produced in the fiscal year ending June 30, 1966.

TABLE 9.—Tissue virus vaccines and number of doses produced in fiscal year ending June 30, 1966

Animal tissue virus vaccines	Doses (fiscal year 1966)
Cattle: Total doses	510, 000
Wart vaccines inactivated. Papovavirus—bovine papilloma.	
Dog: Total doses	8, 500, 000
Canine distemper LVM	1, 330, 000
Canine hepatitis LVM	NA*
Rabies LVM	7, 170, 000
Sheep: Total doses	970, 000
Bluetongue LVM ungrouped arbovirus.	
Swine: Total doses	3, 350, 000
Hog cholera LVM.	
Poultry: Total doses	7, 577, 000, 000
Avian encephalomyelitis LV Picornavirus.	31, 000, 000
Bronchitis LV Possible myxovirus.	2, 961, 000, 000
Fowl laryngotracheitis LV Herpesvirus, subgroup A.	163, 000, 000
Newcastle disease LV	4, 112, 000, 000
Fowl pox LV	236, 000, 000
Pigeon pox LV	74, 000, 000

*Not available.

From our current yearly production and use of 176,800,000 doses of cell culture virus vaccines and 7,590,330,000 doses of animal tissue virus vaccines, we have no field report of clinical evidence of oncogenicity or tumorigenicity in the animals used in this country. In evaluating this negative statement it is necessary to consider several facts that limit its significance:

1) Oncogenicity and tumorigenicity require the presence of large enough numbers of viral particles or cells in a host made susceptible by complex, genetic, chronological, physiological, pathological, biological, chemical, or physical factors.
2) We have no report of any attempt to correlate the incidence of animal tumors with the use of cell culture vaccines.

3) The life of cattle, sheep, swine, chickens, and turkeys is most generally shortened because they are slaughtered. Few pet horses, dogs, cats, and birds are kept to live through years of senility during which their immunologic competence may wane and yield to oncogenic viruses or tumorigenic cells.
4) Possibly maternal antibodies protect animals through their neonatal period, when their immunologic competence is developing, until they reach the age of natural resistance, during which oncogenic agents or tumorigenic cells produce active immunization.
5) Piedrafita (*1*) in 1951 reported an unusually high incidence of fowl leukosis in Spain following the use of killed Newcastle disease vaccine.
6) Burmester *et al.* (*2*) in 1956 reported transmission of visceral lymphomatosis virus in live Newcastle disease vaccine.
7) Payne *et al.* (*3*) in 1966 reported contamination of egg-adapted canine distemper vaccine by avian leukosis virus.
8) Grass *et al.* (*4*) in 1967 reported several outbreaks of infectious sinusitis in turkeys, believed due to fowl pox vaccine contaminated with *Mycoplasma gallisepticum.*
9) Sarma *et al.* (*5*) in 1967 reported the induction of tumors in hamsters with infectious canine hepatitis virus.
10) One cannot help thinking that the extensive losses of our poultry industry to avian leukosis could be associated with the extensive use of vaccines.
11) Huebner (*6*) in 1957 proclaimed that "Guilt by association is as invalid in virology as in the halls of justice."
12) Koch (*7*) in 1884 and Rivers (*8*) in 1937 postulated that a microbe found in suspicious circumstances should be regarded as "innocent until proven guilty."

The above proclamation and postulate appear too comfortable for the hidden suspect. I suggest that the most innocent looking be considered a possible suspect until proven not guilty. Under these premises, cell cultures may be used to produce good virus vaccines.

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Experience With an Established Cell Line for Veterinary Vaccine Production

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Dr. LaSalle and I are in substantial agreement on the use of established cell lines for production of veterinary vaccines. It seems unnecessary to comment on his statistics and I will tell you about a veterinary vaccine prepared on an established cell line, which has been commercially available to veterinarians since 1963, how it was developed, and what has been learned from working with it. I believe I am in the unique position of being the only person in this room who has had experience of this kind.

In 1963, Norden Laboratories introduced a new vaccine containing two modified live viruses propagated on an established cell line for the protection of dogs against canine distemper and infectious canine hepatitis. The product has the trade name of Enduracell D–H. Since then, approximately 1 million dogs have been vaccinated with this product. It is difficult to state precisely how many individual dogs have been vaccinated since we recommend a two-dose schedule for puppies and there have probably been a substantial number of dogs that received annual booster doses.

The cell line we use is of canine kidney tissue origin. It was received from Dr. Erling Jensen of Smith, Kline and French Laboratories at the 96th passage. We have continued to refer to the cell line by the unimaginative description, DK cell line. When the technique of freezing cells in glycerin was described, we determined that these cells could be frozen. Since then we have maintained the DK cell line in the frozen state between the 120th and 126th passage levels, except when actually working with it. The cells grow very uniformly in Eagle's BME medium supplemented with 5 or 10% horse serum. From studies made at the 128th and 145th passage levels, it has been determined that the DK cell line has a modal number of 74 chromosomes instead of 78, the normal canine number. It has two X chromosomes and one large acrocentric chromosome which we believe to be a marker chromosome. All of the other chromosomes are acrocentric which is typical of the dog. Observations to date indicate that the DK cell line contains no abnormal chromosomes. A complete report of our studies of this cell line has been accepted for early publication in the *Cornell Veterinarian.*

In 1959, when we first began to work toward development of a cell culture vaccine for infectious canine hepatitis and hopefully canine distemper, it did not take long to learn that contamination of primary canine cells with infectious canine hepatitis virus was a common occurrence. Fortunately this virus produces a cytopathic effect, making it relatively easy to recognize its presence. To avoid contamination, the concept of using an established cell line to provide an unlimited supply of cells for vaccine production seemed desirable.

Some of the advantages and certain potential drawbacks of using an established cell line were not apparent in 1959. Initially, the problem of whether these cells were capable of producing neoplastic growth in animals was not of primary concern to us. Preliminary experiments in dogs and in hamsters indicated that the DK cells did not produce tumors. At the time we applied for a license to produce vaccine, we were asked to supply more data on this subject by Dr. Gerald Peacock of the Veterinary Biologics Division. We then devised a test patterned after Sanford's method using intracerebral inoculation of live cells and cellular residues into mice irradiated by X ray. For this purpose, a passage level 25 passages beyond the master seed stock was selected. It was assumed that in the normal course of vaccine production this passage level would never be exceeded. Later we had an opportunity to inoculate newborn puppies with both live cells and cellular residues and observe the puppies for a period of nearly 4 years. In 1964, it was suggested that we check the DK cell line by the procedure of Foley, which involves the inoculation of cells and cellular residues into the cheek pouch of hamsters treated with cortisone and this was done. The DK cell line appears to have no tumorigenic tendencies. The results of these tests also indicated that cell residues prepared by freezing and thawing or by lyophilizing, steps normally used in vaccine production, have no tumorigenic tendencies even when derived from neoplastic cells. It is our conclusion that providing all viable cells can be excluded from a vaccine and the cell line has not undergone viral transformation in becoming established, any danger from cellular residues producing tumors seems remote.

The incidence of neoplasms in dogs is just a little greater than in people. Although we have not specifically sought statistics on the occurrence of neoplasms in dogs receiving this vaccine in the field, neither have we received any reports that indicate there is any increase. One of our competitors has used this issue to raise doubts about our vaccine, so I am certain practicing veterinarians are well aware of it.

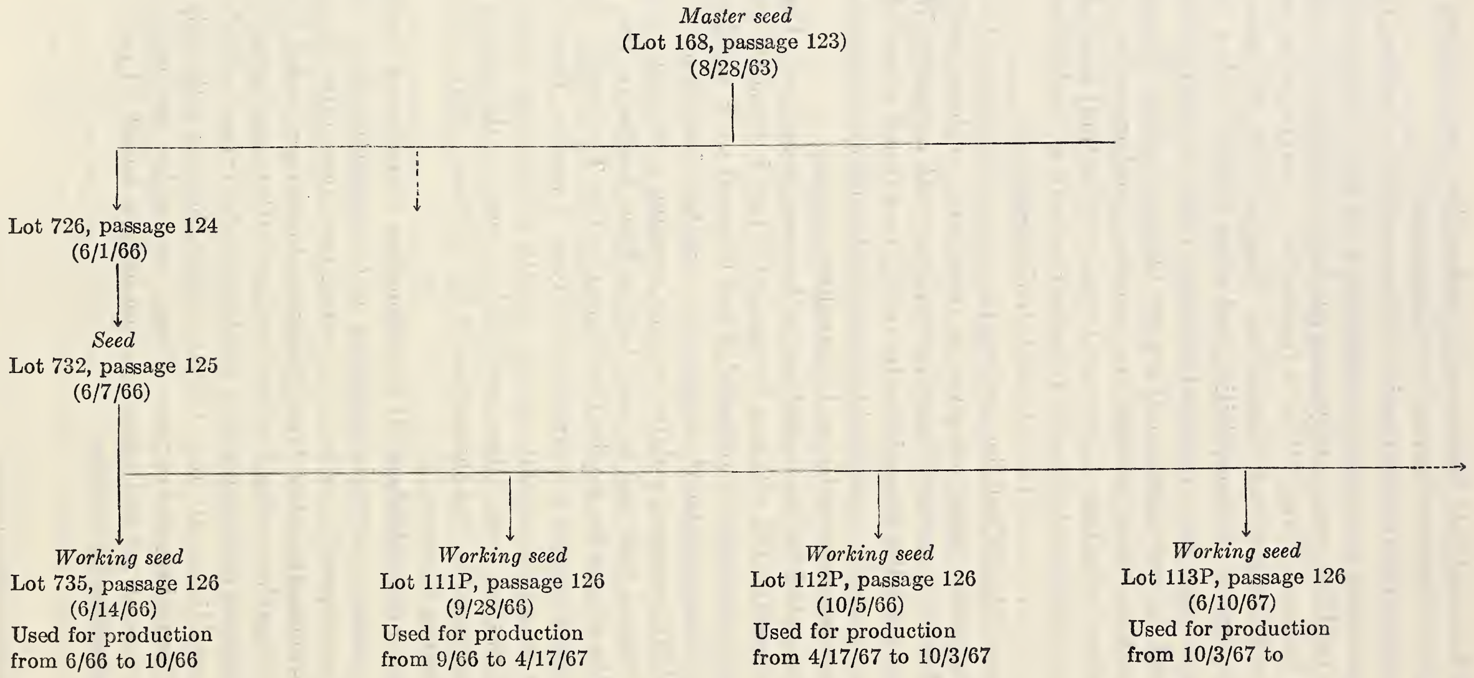
It is my opinion that uniformity of virus production has been the greatest asset derived from use of an established cell line. Theoretically an established cell line contains only one type of cell and every cell is genetically related. Because of this, within the limits of biological variation, each individual cell has the same biochemical characteristics, the same growth rate, the same virus susceptibility, and when infected with a virus will respond the same way. Growth of viruses by other procedures is relatively haphazard. A virus-infected cell is a new biological entity. The attempt to grow a well-characterized virus in a completely uncharacterized cell (this may be in an animal, an embryonated egg, or cell culture) does not lead to a uniformly predictable result. To make the result as predictable as possible, both the virus and the cell need to be characterized.

The use of the DK cell line for growing canine distemper and infectious canine hepatitis viruses has given us remarkably uniform results. Each serial of vaccine completes its production phase with regularity. When our procedure has fallen down, it has invariably been traced to lack of control over factors usually considered to be standard conditions, such as the incubator temperature or deviations in the culture medium.

Every serial of vaccine is produced at the same passage level. Text-figure 1 shows how the DK cell line is managed. There is a master seed stock from which seed stock is prepared. From this seed stock working seed is prepared. From each lot of working seed a number of serials of vaccine are prepared. To prepare a serial of vaccine, working stock is taken from the freezer, thawed, and used to plant bottles. These bottles are then used to expand the cells to the required number of bottles. The number of expansion passages remains constant for each type of vaccine. Therefore, each serial of vaccine is actually grown on cells at the same passage level. Recently this has been further controlled by counting the number of cells which are passed so that the number of cell divisions within each bottle is approximately the same.

The use of an established cell line technology controls and standardizes vaccine production from the beginning of the production cycle to the end rather than depending entirely on control procedures after the serial of vaccine is finished to detect flaws. This concept is being extended to the production of a rabies vaccine for dogs, cats, and cattle using the same cell line. It is anticipated that eventually it will be possible to prepare all of our vaccines on established cell lines. I firmly believe that this is the only way to proceed from where we are now—where virus vaccine production is some place between an art and a science—to a modern industrial process with resulting benefits for everyone.

VIRUS VACCINE PRODUCTION


Master seed
(Lot 168, passage 123)
(8/28/63)
Lot 726, passage 124
(6/1/66)
Seed
Lot 732, passage 125
(6/7/66)
Working seed
Lot 735, passage 126
(6/14/66)
Used for production
from 6/66 to 10/66
Working seed
Lot 111P, passage 126
(9/28/66)
Used for production
from 9/66 to 4/17/67
Working seed
Lot 112P, passage 126
(10/5/66)
Used for production
from 4/17/67 to 10/3/67
Working seed
Lot 113P, passage 126
(6/10/67)
Used for production
from 10/3/67 to

TEXT-FIGURE 1.—Management of stock cultures of established canine kidney cell line for vaccine production.

Experience With Vaccines—General Comments

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It is rather like a symphony with slow opening movements. This 3-day session has reached a crescendo this morning, and it is very difficult to follow some of the speakers that were on the agenda this morning. However, it seems to me that a number of significant points that are not likely to be resolved at this session need to be thought about and brought to a point of resolution, certainly in the near future if any effort is to be made to bring a rational basis for human virus vaccine production under scrutiny.

The first of these was mentioned several times earlier. It has to do with follow-up of individuals immunized with preparations of the various sorts currently available and of preparations likely to be available in the immediate future. Dr. Robbins likes the idea of a prospective study of vaccinees.

Dr. Perkins' committee raised the very valid business of establishing cause and effect when effect or potential effect is measured in months or years beyond the time of the potential cause. Yet, it would seem well worthwhile to look at ways and means of obtaining this information, not only within the framework of the human subjects that are being administered these vaccines, but also the animal subjects that are receiving, if my calculations are correct, vaccines several orders of magnitude more frequently. The idea of multiple cell sources for vaccines is obviously a desirable one. The limited numbers of cells that have had extensive "safety" testing in laboratories today, at least for use in vaccines for potential human use, obviously need to be extended.

Finally, I would like to introduce a concept of my own. I am sure other people have thought of this. It has been in the minds of many of us who are concerned with the use of vaccines. It has to do specifically with changing of precedent or, at least, considering changing of precedent. Heretofore, approval of the use of vaccines has depended upon the issue of a commercial license. And this has inferred the production of a preparation that can be used without discrimination by the medical confraternity at large. In other words, it has to be a preparation of universal usefulness and universal safety.

I should like to raise the question whether it is appropriate to consider limitations of this generalization. Not every preparation, not every antigen, has the same potential usefulness or significance in all human populations. How this would be engineered administratively and legally I have no way of knowing. Yet, the idea from a scientific standpoint is attractive to me. And this leads me in part to a response to Dr. Rubin's question earlier and Dr. Koprowski's very prompt recognition that the control of acute respiratory disease is likely to be achievable—the eradication is not—by the use of vaccines.

The rationale in the present suspension of the use of adenovirus type 4 vaccine in military populations stems from the fact that there is no significant adenovirus 4 disease to protect against, just as, for example, there is no significant paralytic poliomyelitis to protect against in military populations at this particular time.

When one thinks of the stresses that a recruit is subjected to, one automatically thinks in terms of attempting to reduce these. And one of these stresses has to do with the application by injection or ingestion of foreign proteins. Therefore, until such time as there is epidemiologic evidence that type 4 virus is, indeed, present on basic combat training posts and is causing disease, there is no need to use this vaccine, at least in the way it has been used in the past.

Now, this doesn't mean that it won't be used. It simply means that our ingenuity at the moment is taxed to determine how best to use it. And I am sure there are some of you who know that one of our efforts at the present time revolves around using vaccine virus in previously immunized individuals, vaccine virus applied to the upper respiratory tract, the nose specifically, as a means of establishing respiratory infection without disease in units of men.

The aim here is to determine whether such established upper respiratory tract infection with the vaccine virus type 4 will interfere in any way with the introduction and transmission of type 7 and type 21, two agents that we know have an epidemic potential in orally immunized adenovirus type 4 individuals. The tests are under way. They obviously cannot be escalated beyond the rates that scientific integrity dictates. Suffice it to say that if the initial studies which involve small numbers of men establish that this, as a technique, will work there will be a broader application for the use of adenovirus type 4. It is appealing to me as a biologist because if it works for type 7, it should work for the other adenoviruses that presumably are involved. And the problem of chasing down yet another antigen with all of the attendant difficulties in establishing safety standards is minimized.

There are those of you who will say that this is a fond hope. I certainly will agree with such individuals and agree to that premise. However, at the moment, it is the only reasonable approach that is available for the control of acute respiratory disease in military populations in the next year or two.

[illegible]

Experience With Vaccines—General Comments

Thomas Francis, Jr., *Department of Epidemiology, The University of Michigan, School of Public Health, Ann Arbor, Michigan 48104*

If I were a little smarter, I would pass, but I can't always have the opportunity to express a few views under these circumstances.

I am not sure whether I am not in the camp of the Philistines at the present time because there seems to be a general assumption that virus vaccines should be live vaccines and that this is completely acceptable. I still would like to retain a demurrer on this and to feel that there is no single thesis that is completely right.

And perhaps I am reminded of the story that I heard recently of the man who was trying to decide on a watch to buy—he was a watch collector, and there were two watches, quite valuable. One of them didn't run, and the other lost a second per day. So he went to a computer for advice, asking which he should buy. And the computer said: "If you buy the one that is stopped, that doesn't run, at least it will be right twice a day. If you buy the other one, it will only be right once every 120 years."

Apparently he never thought of the idea that you could adjust the situations. But at least, that was his point of view. One of the arguments for the live vaccine thesis is that one can't improve on nature and, therefore, one should mimic it.

Dr. Koprowski spoke of precedents earlier. And we certainly are following the precedent from the celestial kingdom on down when pox sowing was a common procedure in the live vaccine. The effort to introduce live vaccine for smallpox was established then. So we had a long period of precedence for this outlook. It is my belief, however, that in the advances of science and medicine, one should try to improve on nature. And it is for this reason that I think we shouldn't be bound to that concept.

There is another point commonly used in the consideration of live vaccines. That is, one gets local immunity in certain areas as well as a general immunity which reinforces the resistance. And I don't think it has been established in any concrete fashion that there is a specific cellular immunity derived from the infectious process, whether it is by natural means or otherwise.

I would like to recall the fact that many years ago when we were studying the question of influenza vaccine, we undertook the problem of infection of human subjects with not too highly attenuated strains, and they took sick. Three or four months later, we reinoculated these people with the same strain of virus, and again about a third of them who had been sick the first time took sick the second time.

Now, certainly this didn't convince us that we were having a longer term immunity because people who had been vaccinated subcutaneously at the same time, if anything, had a better resistance than the ones who had been previously infected. So there again, the idea that you have a local antibody as being a major factor, has not convinced me.

There is one thing I would like to say further regarding this meeting. I am very pleased to see that the scare has been reduced so far as the matter of the oncogenic viruses in relation to vaccines is concerned. I hope that this does mean that one can under reasonable circumstances use materials which may have some theoretical objections, but where this objection cannot be substantiated, particularly where there is a need for those vaccines. I have never been one who has been particularly scared by the theory if one could not find substantiating information.

Now, one of the principles of prophylactic immunization, at least with virus vaccines—and this has been repeatedly emphasized in the last 2 days—is to have a vaccine that contains the agent you wish to have as immunizing agent and not a lot of others. I think this can be further clarified, and I think this applies to contamination of cell lines as well as it means contamination with other viruses or other agents. It seems to me the studies that Dr. Anderson described yesterday are perfectly fine examples of how this can be done and should be done to arrive at the desired degree of purity.

Many of these problems have been discussed and many of the worries and cares and great efforts can really be reduced or minimized if one turns to the idea of inactive vaccines, selectively inactivated to retain the proper potency of the immunizing antigen and yet to eliminate many of the other things. This is really to reemphasize what Dr. Gard said last night, and it is not a new subject at the present time.

Dr. Perkins spoke of improving safety. Inactivation is one of the ways to do it. Dr. Hull spoke of the inactivation of the carcinogenic activity of cells directly by inactivation procedures.

Furthermore, it seems to me that the line of future immunology for man and preventive immunology should be working toward purified antigens rather than sticking to the primitive concept of continued and repeated infection, especially with the multitude of disorders with which we are confronted. There is much greater likelihood that one can put together the purified antigens or the separated antigens which are prepared, purified, and specifically identified and are stable, put these together into a composite type of vaccine which can give a basic immunity early in life rather than depending on this long series of expected acute infections that are not completely certain.

So my hope is still that we shall move toward the field of the purified antigens and work on the future of immunology rather than simply be held to precedent.

I suppose it is a little ironic that about 13 years ago at a conference in Rome, there was discussion about the possibility of poliomyelitis vaccine. I happened to be chairman of the panel that was being held. Dr. Koprowski was one of the speakers. The situation was a little reversed; the themes are still the same. At that time, he was calling for the support of precedent, stating that we were living in the era of Pasteur, Jenner, and Tyler. He didn't mention the celestial kingdom as being part of the earlier group we were following.

At that time I remarked that I happened to come from another school that belongs to Avery, Heidelberger, Landsteiner, and others who believed in the purified antigens. Our situations haven't changed much.

Experience With Vaccines—General Comments

JOHN P. FOX, *Department of Preventive Medicine, University of Washington School of Medicine, Seattle, Washington 98155*

MANY, if not most, of the points that I had in mind have been well covered and I will only try to underline some of them. But first I would like to pick up the point which Dr. Francis just reopened and which Dr. Gard spoke to so cogently last night. I am a person who, traditionally, has been a live virus man rather than a killed virus man. But I do agree that, in terms of the long-range objective, one certainly should look at the production of vaccines which contain only that which is really essential to do the job of immunization, whether this be the intact virus particle or some fraction of that which contains the antigen specifically concerned with protection.

Now, this, I think, is for the future. We must deal with the present situation in the meantime, and that brings us back to the basic problem of working with the kinds of tools that we presently have and attempting to improve these as quickly as we can.

Yesterday's papers in particular outlined a rather worrisome array of actual and theoretical problems and also presented some solutions, at least partial, to these problems. Today's papers are further reassuring with respect to the general problem of oncogenic viruses in all vaccines and with respect to the experience with diploid and heteroploid cell vaccines.

I thought I would like to say a little bit more about the experience that we have had with two very widely used live virus vaccines; namely, yellow fever and live poliovirus vaccine, and speak here with reference to aspects other than those related to the viruses that the vaccines are supposed to contain. I think the problem of the efficacy of the vaccines is not what is at issue here.

The potential importance of nonviral antigens is limited to parenterally injected vaccines and is greatest for those depending on nonmultiplying viral antigen while the problem of undesired microbial flora, including possibly oncogenic viruses, is more pressing when the essential viral antigen is infective.

Two types of yellow fever vaccine, French neurotropic and 17D, are produced in the mouse brain and chick embryo, respectively. Because they are not produced in cell culture, they are particularly vulnerable to the problems of present concern. Postvaccination encephalitis due to the vaccine strains has plagued both, but especially the former. Problems relating to nonviral antigen have been (and should be) minimal because of the relatively high dilution of the vaccine inoculated. Nonetheless, rare instances of allergic reaction due to egg yolk sensitivity (*1*), systemic allergic (*2*), and serum-sickness type reactions (*3*) have followed the use of 17D vaccine. It also has been blamed recently for inducing sensitivity to subsequently inoculated duck-embryo rabies vaccine (*4*). The major problem, encountered in Brazil (*5*) and the U.S. armed forces (*6*), was with serum hepatitis and represented the consequences of a contaminant derived, not from the source of virus, but from an additive, human serum, the use of which has long since been discontinued.

Chick embryos often contain an avian adenovirus (CELO) and, except when the chickens are from special flocks, universally contain viruses of the avian leukosis complex, the latter being a known contaminant of 17D vaccine. While cells of human and simian origin can be infected in culture with Rous sarcoma virus (a member of this complex) (*7*), neither children nor adults (*8*, *9*) developed antibody to these viruses when given vaccine demonstrated to be contaminated. Thus, in an experience extending over a 30-year period and embracing many millions of vaccinees, no evidence of trouble attributable to undesired viral or other microbial flora of the virus-producing host has been uncovered.

The situation with respect to live poliovirus vaccine, at least until very recently, was nearly equally reassuring. This has been by far the most widely used vaccine for man produced in cell cultures (of a class, primary cell, not included in the program of this session). Kidney cells from rhesus or green monkeys have constituted the production hosts and are the recognized potential hosts of a large number of viruses, most of which, including the very dangerous B virus (*10*), have been excluded successfully from the vaccine.

This success, of course, depends on knowledge of the existence of the viruses and availability of means for their detection. Thus, until it was recognized rather fortuitously (*11*), SV40 was universally present in the Sabin vaccine and given to a vast number of persons. It does induce infection in man after feeding (*12*, *13*), after respiratory infection (*14*), and after parenteral injection and antibody persistence after such injection is compatible with viral persistence (*15*). Indeed, a serologic survey in India suggests that in regions of high monkey density, natural infection in man with SV40 virus occurs (*16*). Although impressively oncogenic in hamsters (*17*), no similar effect has been observed in man. That it probably will not be observed is suggested by the failure of infected children to develop antibody to the SV40 tumor antigen (*15*).

The recent association in Europe of green monkeys with a still uncharacterized (possibly rickettsial) agent highly pathogenic for man is of course most disturbing. However, its overall importance must be evaluated in the light of the very extensive prior use of live poliovirus vaccine with which the only recognized adverse effects have been related to the vaccine polioviruses themselves.

Both the SV40 story and the recent green monkey episode illustrate a major general problem of live virus vaccines. Unless or until the presence of an adventitious agent becomes manifest, it is not possible to exclude it. This is true regardless of the origin of the cells used in vaccine production, although the possibility of exclusion should be significantly greater when, as with diploid cell lines, the cell population can be studied over many generations. However, this uncertainty must not be given undue weight. The long and extensive experience with live virus vaccines produced in the intact host or in primary cell cultures has been remarkably free from adverse effects attributable to adventitious agents derived from the virus-producing host.

I also would like to come back to a point which Dr. Miller and Dr. Robbins made and which Dr. Buescher has already made reference to. That is the problem of follow-up of experience after vaccination.

I would like to emphasize again the great desirability of the prospective type of approach and suggest that, in effect, this kind of an approach may yet be possible within the specific case of yellow fever vaccine as used in the military services. I have been laboring under the delusion that all troops got yellow fever vaccine during World War II, at least after the hepatitis problem was cured. I am informed that this was ultimately administered only to troops who were actually going to areas in which yellow fever virus was known to be present. If such is the case, it would be possible to constitute, retrospectively, groups of soldiers, sizable groups, who were comparable in essentially all other respects except that they did or did not receive yellow fever vaccine and to scrutinize their experience, not only for problems of oncogenesis, but for other problems as well, including any related to the presence of serum hepatitis virus.

Earlier in this session I made a remark on an opportunity that exists with respect to measles and I would like to broaden that just a little. The point that I was trying to make was that the very great acceptance of measles vaccine currently is going to nearly eradicate, if not completely eradicate, clinical measles from this country; that we do not know all of the implications of measles currently, and that we have an opportunity to learn something more about the natural history of measles by constituting groups now that we can identify as having had measles or who will not have measles because they have had vaccine. I was talking about such perhaps trivial factors as learning ability and behavior. Dr. Koprowski mentioned to me another item, namely, the possible relation of measles to multiple sclerosis. This is something which may ultimately become evident as a result of studies of this sort.

VIRUS VACCINE PRODUCTION

I had planned to, and I think I will, close with one other remark which is an echo of something else that I have heard. Obviously, no biologic is utterly safe. And in any case, its use must be justified by weighing the ascertainable associated risk against that resulting from its nonuse. And the specific remark that I planned to close with is a quote from a paper by Dr. Hilleman (*18*). So he is getting the last word after all.

"While it is axiomatic that vaccine development should be subservient to safety, it is likewise essential that concepts of safety should be founded on fact and seasoned judgment rather than on theoretical or conceptual phobias that preclude further progress in this approach to the control of infectious disease."

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GENERAL DISCUSSION

Dr. M. R. Hilleman: I should like to make a few comments which I think might be pertinent to the subject.

Dr. Buescher has touched on the matter of the difficulty of preventing respiratory disease and this is certainly true. There are, however, new developments which indicate that such objective is being realized. Eaton agent, or *Mycoplasma pneumoniae*, vaccine has now reached the stage of showing high-level efficacy and, hence, control of this agent is on the horizon.

Parainfluenza virus vaccines now induce levels of antibody as good or better than those for influenza. All we are left with is the need for obtaining proof of efficacy under field conditions.

Influenza virus vaccines can be very good, if the proper strains are in the vaccine. The only one of the pediatric viruses which remains a problem is the respiratory syncytial agent.

There are good killed vaccines against adenoviruses and now good live vaccines against these agents. However, because of the carcinogenicity problem, there is limitation to use of live type 4 adenovirus vaccine, which Dr. Buescher hopes might provide some protection against other adenovirus types if given nasally. I think this is an approach worth exploring, but I wonder to what extent such vaccine might open the way to secondary bacterial infections and cause problems of guilt by association if not guilt by cause. I think those of us who have the responsibility and liability in use of vaccines would tend to be very cautious in introducing live viruses routinely into the respiratory tract for immunization purpose.

In seeking control of viral disease, one really should look to all possible measures and should use the best approach for the particular virus that one is trying to control. Killed vaccine may be best for one virus and live vaccine for another and so on.

Though this session is dedicated primarily to vaccines, I should like to mention that another approach is fast rising on the horizon: the interferon mechanism. Vaccines generally give long-term immunity, and this is their advantage, but such immunity is narrow spectrum. Interferon, by contrast, is extremely broad spectrum but the immunity is of only short duration.

Control of rhinoviruses seems unlikely by vaccination. The fact that rhinovirus serotypes probably number in the hundreds makes this not feasible from the economic as well as the biologic sense. The interferon approach offers far more promise. There are two approaches to interferon utilization: One is through administration of preformed exogenous interferon; the other is to induce the body to make and distribute its own interferon.

Our own work and the work of others with exogenous interferon in animal models have shown that, while such administered substance can produce a favorable local or systemic effect, it shows little promise in the practical sense. I once calculated that in the most ideal system we know of, which is egg interferon against Eastern Equine Encephalitis virus in chick-embryo cell culture, 40 mg of interferon would be required for an equivalent minimal human dose. Preparation of such material would require 2,000 eggs, which would be costly.

This has led us to the approach of interferon inducers letting the body make its own interferon. During the past months, we announced the finding of what apparently is the alarm system for the natural induction of interferon in RNA virus infections. This is double-strandedness of the RNA and appears to be the basic mechanism or the basic requirement for interferon induction by such agents.

There are several kinds of double-stranded RNA which have been used in our work. One of these is of synthetic origin and consists of a complex of the isosinic acid and cytidylic acid homopolymers. Another is double-stranded RNA which we recovered from extracts of *Penicillin funiculosum* and which is commonly referred to as helenine. The activity may derive from a viral contaminant of the fungus. A third source is from the virion of reovirus 3 which uniquely contains double-strand nucleic acid rather than single strand. Finally, replicative form double-strand RNA from coliphage has been used. All of these are active in microgram amount in inducing interferon and resistance to viruses *in vivo* and *in vitro.*

These materials are highly active when given nasally, and this approach offers special promise for control of viral respiratory illnesses, particularly the common cold.

Dr. W. Hennessen: Mr. Chairman, I would like to make a remark which I thought somebody else would make after the veterinary papers. I should like to draw the attention of the audience to the fact that there is a vaccine that has been in use since 1965 in the veterinary field against foot-and-mouth disease which is prepared on BHK-21 cells, and the cells are oncogenic.

It is a low guess, I should say, that until now there have been 20 million cattle immunized with this vaccine in parts of Europe, Latin America, and Africa as well. And so far no untoward effects have been observed regarding malignant tumors from this vaccine.

Dr. N. C. Reisinger (National Cancer Institute) : I think in regard to this foot-and-mouth disease vaccine being inoculated into 20 million or more animals, that it possibly could have, but I don't say it has, increased the tumor frequency or incidence in these animals by a factor or two. Since we don't have too adequate a baseline in many countries on this, we would not know it.

Dr. H. Koprowski: Yes, I think this point is pertinent to Dr. Buescher's remark of inhibition of epizoological surveillance, similar to that proposed for man. I know that efficiency of this procedure may not be as good for animals as for man.

Dr. F. C. Robbins: In this regard, I wanted to ask the people who deal with animals whether any of the animals live long enough or are allowed to live long enough so that you would expect to really have an opportunity to survey for particularly oncogenic viruses.

Dr. B. LaSalle: I said that the life of cattle, sheep, swine, chickens, and turkeys is abbreviated by slaughter. And after any cattle have served their economic usefulness, they are disposed of. I cannot tell you how many cows, for instance, live to the senile age of 20 years, but I am sure that very few do comparative to the human population. I don't have any figures on this.

Dr. Reisinger: What about dogs?

Dr. LaSalle: Since dogs are pets, they are kept longer, but there, again, I don't have any figures on the dog population that is kept to senility and dies of old age. I would guess maybe 5% but I may be way off.

Dr. Reisinger: In our study of spontaneous cancers in cattle in this country, with the exception of epithelioma of the eye, 90% or more of the tumors that we have studied in packing houses all over this country, in federally inspected plants, occurred in cattle 5 years or older. And approximately 20% of the cattle in this country are slaughtered at the age of 5 years and older. In some European countries, of course, this percentage would be much higher.

Also, the frequency of tumors in slaughtered animals varies according to a rather complicated economic cycle directly related to how many old cows are slaughtered and how long they have been kept back before being slaughtered.

Dr. M. Rosenbaum (Great Lakes Naval Training Station, Great Lakes, Illinois) : Since 1956, Naval Medical Research Unit No. 4, Great Lakes, Illinois, has conducted controlled vaccine evaluation studies in large populations of naval recruits. In several instances parenterally administered inactivated virus vaccines produced in monkey renal tissue cultures have been associated with significantly more cases of clinically diagnosed rubella than was found in subjects who received placebo. The vaccines included adenovirus types 3, 4, and 7, rhinovirus type 1, and Salk poliovirus types 1, 2, and 3. All vaccines were prepared in monkey renal tissue cultures and viruses were inactivated by formalin treatment. Placebo control subjects received only tissue culture growth media with formalin concentrations similar to those of the vaccine preparations. Attempts to provide a biological placebo, *i.e.*, aliquots of the uninfected tissue culture used to propagate the vaccine viruses, have been unsuccessful due to technical considerations of manufacture.

Although the vaccines generally have been effective in protection against the specific viral diseases they were directed against, in each study, subjects who received these vaccines had an excess amount of clinically diagnosed rubella.

Confirmation of the clinical observation by rubella serologic tests (HI) is now under way.

The data in table 1 show the number of studies and rubella incidence rates per 1,000 men for vaccine and placebo groups. The excess rubella in vaccinated men has varied from 6 to 38%. The consistency of these data and the large populations involved substantiate their significance.

Table 1.—Incidence of clinical rubella in naval recruits after parenteral inoculation with various viral vaccines produced in monkey kidney cell cultures

Year of study	Vaccine type	Number treated	Number of cases	Rate/+ 1,000	Excess of rubella in vaccinees (%)
1962–63	Adenovirus.............	15, 654	641	40. 9	12. 2
	Placebo................	5, 132	184	35. 9	—
1963	Rhinovirus (type 1A)....	1, 384	65	47. 0	6. 0
	Adenovirus (types 3, 4, and 7).	928	53	57. 1	22. 6
	Rhinovirus + adenovirus.	919	66	71. 8	38. 5
	Placebo................	1, 357	60	44. 2	—
1964	Poliovirus (types 1, 2, and 3).	1, 035	78	75. 4	27. 1
	Placebo................	1, 054	58	55. 0	—

The cause of this phenomenon is not known. However, several possibilities have been considered: (*a*) presence of latent agents in the monkey cell culture or in the viral seeds, (*b*) removal of interference mechanisms by the effect of the vaccine, or (*c*) hypersensitivity reaction to the cell culture antigens.

Dr. W. Hammon (University of Pittsburgh): I would like to ask a question in regard to WI–38. A great deal of attention has been devoted to that in the meeting. Dr. Perkins mentioned earlier that he thought this was one that we might be ready to go on. It might come out of this meeting with clean skirts, but I think nobody has discussed the arithmetical quantitative possibilities in this one cell line. It has taken a number of years to get together all the necessary data in respect to this one cell. It doubles on transfer, apparently being used somewhere between the 20th and maybe the 35th passage. And if every vaccine manufacturer essentially in the world began to use it, how long would it last?

Dr. L. Hayflick: If you do the necessary calculations, which I won't bother doing now but which we have published, you find that, when the original population established from the primary embryonic tissue is multiplied by 2^{50}, the upper limit of cells produced from the original tissue would be in the neighborhood of 20 million metric tons (Hayflick, L., and Moorhead, P. S.: *Exp Cell Res* 25: 585, 1961).

Now, the cells, of course, are not recommended for use in vaccine production beyond the 30th or 35th passage, so that this quantity is considerably reduced. I think a more realistic answer, however, would be to say that in 1962 we originally produced about 700 ampuls of WI–38 at the 8th passage level and banked them in liquid nitrogen. Now, 5 years later we have 500 ampuls left. We have distributed WI–38 to hundreds of laboratories throughout the world and only now has the demand for these cells begun to drop off considerably, since most people who regularly work with them have produced their own banks of cells. When we give investigators seed material at a low passage, say, 10th or 11th passage, these individuals work the cultures up to the 17th, 18th, or 19th doubling level and make their own banks. So regardless of what objections there might be to obtaining the theoretical astronomical figure, the practical aspects are that, at the current rate of use and what we can project for the future, I see absolutely no danger of running out of WI–38.

Perhaps, in closing, I should mention my support for the statements made by earlier speakers that we should certainly not rely on a single diploid population for the production of all virus vaccines. I would urge, however, that the other cell populations contemplated would come from the same kind of source material as did WI–38.

Dr. B. A. Rubin: Perhaps Dr. Hammon would be interested in a practical production experience. At least 5000 liters of vaccine were derived from 2 ampuls of 11th passage material from Dr. Hayflick. And I am quite sure that we still have lots of it left. We subcultivated it to the 16th passage where we froze away our own ampuls. If Dr. Hayflick would occasionally supply us with another ampul, we could go on for quite a while.

Session VI

Summarization

Chairman: Donald J. Merchant

Characteristics of Cell Culture Systems: Primary Cultures, Diploid Cell Lines, and Continuously Propagated Cell Lines—Summary

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FIRST, I express my thanks to Dr. Roderick Murray and Dr. Donald Merchant and the Organizing Committee for a very fine Conference. Dr. Harris first emphasized that modulations (phenotypic modifications) in cultivated cells may be a prompt response to changes of environment. At times these fluctuations are more subtle and are expressed over many cells generations. At other times they seem to be of a permanent character, but they eventually prove to be reversible.

As demonstrated by the existence of both diploid and mixoploid cell lines, the more permanent genetic changes are of long-range consequence. These changes can be demonstrated by karyology, serology, or biochemical markers. It is interesting to note that several apparently stable lines of diploid cells have been established. WI–38 is one example of such a line, which has been characterized by karyology and other markers.

Distinctions based on proved permanent characteristics were discussed under the headings above. Since it was agreed that the same classes of markers should be applied to diploid, mixoploid, or heteroploid cells, we will here consider the merits of each system for measuring identity or differences.

Dr. Moorhead regarded karyology as a somewhat crude but convenient and critically useful tool. All the gross differences related to a change in chromosome number or morphology are readily recognized. Problems arise due to artifacts and to subtle differences in morphology which make the microscopic work tedious or the results erroneous. Decisions may be impossible if the cells remain diploid despite a fundamental change in marker that does not influence chromosome morphology.

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[1] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

There seems to be a consensus that typical ratios of diploid cells must be demonstrated periodically during successive passages throughout the lifetime of a cell line. We also interpret the sense of the investigators to be that atypical ploidy is permissible provided it is temporary. Since, *in vivo*, atypical ploidy may occur up to the 10% level, we assume that mixoploidy must exceed this level to be a sign that there has been a deviation from the norm. In short, karyology is of major importance for the detection of evolutionary alterations as cell propagation proceeds and also for the detection of accidental cell contamination.

Dr. Moorhead emphasized the limitations of karyology by mentioning a diploid appearance in a malignant tumor and by referring to a well-known fact that mixoploidy is not a guarantee of neoplastic potential.

Dr. Stulberg and Dr. Brand have described elegant methods of identifying the species of origin of cells in both diploid and established cell lines. The evidence that contaminating cells occur in presumably homogeneous cell lines grows stronger each year. In view of the precision and convenience of fluorescent or hemagglutinating antibodies, which combine with cell surface antigens, there is little excuse for neglect of an extremely useful tool.

The demonstration of subtle differences between cell lines from the same species is feasible by means of several well-established principles.

Although a series of biochemical markers are known, Dr. Ruddle used the findings of isozymes to illustrate the fact that the protein carriers of enzyme prosthetic groups in one subline of cells can differ from those in a related line. Five enzymes were used to demonstrate this point. It was shown that isozyme markers in heterozygous configurations can provide information on genetic alterations which evolve in *in vitro* populations. Although Dr. Peterson noted that diploid lines and quasidiploid lines possess the B isoenzyme form of G6PD, mixoploid cell lines possess the A isoenzyme form irrespective of racial origin. Serology offers a second principle for demonstrating subtle differences in proteins for other antigens in related lines of cells. The aim of the tools described by Dr. Brand is to show identity or differences between antigens that may not be prominent in cell surfaces. This purpose is accomplished by immunizing with a battery of extractable antigens. Immunodiffusion of these antigens against the corresponding antibodies affords a means of recognizing differences between antigenic configurations. Characterization of certain antigens was aided by their differential susceptibility to heat, acetone, enzymes, etc.

By means of fluorescent-tagged antibody against soluble antigens, it was shown that antibody may react with deep-seated antigens in the Golgi region. This adds a morphologic parameter to the identification of cell components by means of serology.

The information assembled at this Conference makes it evident that more than one class of criteria will be required to characterize a cell line. Different problems or different situations will select the appropriate combination of methods.

Relative to the technology of cell and virus production, progress in the science and in the art of propagating cells and viruses has been made in several directions. The three most favored physical arrangements for cultivating cells are media flowing over cells on solid surfaces, media perfused through multilayered cell populations, and fluid suspension of cells by stirring, shaking, or sparging.

Although there has been no marked progress in development of cell nutrients, the control of temperature, *p*H, CO_2, oxidation-reduction potentials, glucose and galactose, and lactic acid has made great contributions toward achieving satisfactory production of cells and virus.

Adequate attention has not been given to the primary importance of nitrogen metabolism. It should be recognized that elimination of this deficiency is the pathway toward lowering serum requirements and the attendant hazards.

Dr. Hanks and I believe that thoughtful supplementation of diploid cell lines with known precursors for nucleic acid might offer a means of increasing the replication of viral nucleoproteins. Superior rates of synthesis or turnover of precursors probably explain why established cell lines have given superior replication of certain viruses. The present empirical combination of viral seed stocks, cell culture systems, and physiologic conditions is an exceptionally expensive way of improving the yield of virus. The excellent investment in bioengineering can be made more rewarding by similar investment in the application of present knowledge about synthetic processes.

This report represents the consensus of the rapporteur, Dr. Hanks, and myself to the first session, and he has offered to field the questions after this.

Characteristics of Cell Culture Systems: Viral Flora of Tissue Sources and Tissue and Medium Antigens in Vaccines—Summary

M. M. Sigel *and* Herdis von Magnus, *The Variety Children's Research Foundation, Miami, Florida 33155, and Statens Seruminstitut, Copenhagen, Denmark*

The first part of the session was devoted to the viral flora of animals and cells used for tissue culture. The chicken seems to be the proud possessor of a large constellation of pathogenic and passenger viruses. In fact, according to a speaker, 15 newly recognized myxoviruses were added to the collection among chickens, ducks, and turkeys. Of special importance are those passing through the egg. Among the 15 groups of viruses cited by Dr. Luginbuhl, five deserve special attention as potential contaminants of vaccines or cell cultures used in various laboratory procedures. These are the avian adenoviruses, encephalomyelitis, leukosis viruses, Newcastle disease, and infectious bronchitis. In addition to a real or hypothetical health hazard to the vaccinee or the laboratory worker, some agents may complicate laboratory procedures or production procedures by interfering with the replication of the vaccine viruses.

Although, according to Dr. Barile, *Mycoplasma* contamination occurs with greater frequency in established cells than in primary cultures, at least one species, *Mycoplasma orale*, according to Dr. Rightsel, apparently has been found in primary cultures or early passages of chick embryo fibroblasts where it suppresses the Rous-associated virus, thereby preventing its recognition in the RIF test.

Not all the other groups of chicken viruses have been exonerated. Rather, we lack information about their nuisance value or danger to the cell or vaccine programs. Fortunately, flocks of chickens free of recognized viruses have been developed and should help to minimize the problem.

The dog's viral flora (The word "flora" is bothersome. Should we not break with bacteriological tradition and coin a new term, say "vira," as a third sister to fauna and flora?) appears to be, on the surface at least, less impressive than that of the chicken. This may simply reflect our ignorance, entirely excusable in view of the relatively recent entry of the dog into the tissue culture race. Even so, Dr. Gillespie listed 11 dog viruses, 4 with some oncogenic property. And, as regards the problem at hand, 2 groups, the viruses associated with canine hepatitis and herpesviruses, appear to be of special importance.

In Dr. Kniazeff's view, at least 4 viruses of the cow may find their way into tissue cultures. They are herpesvirus, parainfluenza type 3, enterovirus, and the bovine viral diarrhea (BVD) agent. The problem may be compounded by the extensive use of bovine serum and by the existence of strains of BVD which cause no cytopathic changes in cells.

Dr. Wilner told us of 11 virus infections of rabbits, domestic rabbits and wild rabbits. Yet he knew of no spontaneous infection of cells in culture. But during the discussion period Dr. Duff indicated that he had heard of two or three possible detections of virus, I believe herpes-like virus, in cell cultures of rabbits. One isolate made by Dr. Anthony Nesburn had many characteristics in common with virus III isolated and described by Rivers.

The status of the rabbit cells in culture deserves further study, especially since no one has yet reported oncogenic transformation of established rabbit cells.

It must be stressed that some occult viruses may announce their presence only after prolonged observation. Dr. Hsiung mentioned how she found an agent in horse kidney-tissue culture only after about 80 days of observation. Dr. Melnick made a comment about a similar long-term observation with the measles virus. We recognized an orphan virus in fish cells after 9 months of cell cultivation. While possibly this agent originated from exogenous sources (although the healthy-looking cells cultivated at 20°C were never fed with fish serum), it is interesting that the agent readily goes under cover, giving origin to a carrier culture whose morphologic characteristics, growth rate, and other properties are indistinguishable from the parent culture.

The main part of the session concerned the primate systems, and, because they represent the major substrate for the cultivation of human viruses, primate cells have received the greatest amount of scrutiny. From this has come a recognition of a veritable fog of viruses, not only potentially oncogenic for man but sometimes truly lethal.

Dr. Hennessen's account is new proof that some agents can wreak havoc before they ever get into the vaccine. Twenty-seven severe illnesses with seven deaths are a grim testimony. I am impressed with the concentration of cases, mainly among people who were exposed to monkey blood and monkey tissues as opposed to monkey feces.

It is by no means certain how many viruses are indigenous to the simian hosts and how many are acquired by them from man or other animals.

Dr. Hennessen reports that the vervets implicated in the tragic episode were exposed to a variety of wildlife at the London airport.

The currently available methods can recognize many of these agents. Yet there is a host of diseases, such as hepatitis and those associated with slow-acting viruses, where the tools are inadequate.

In further summation, one suggestion made by a discussant, Dr. Vasington, was of interest. He mentioned serologic prescreening of monkeys before use. By using this approach and screening for antibodies to the foamy agent, he was able to reduce the rejection rate from 36% to 10%. Yet, as was pointed out by Dr. Kalter, some simian viruses are poor antigens, and therefore serologic tests would not necessarily detect infection in all instances.

Dr. Kalter brought out the possibility of using fetal monkey cells which would presumably be free of some of the fecal viruses which contaminated adult tissues. Unfortunately, little is known about the floral (or vira) of the monkey fetus. Moreover, as we all know, monkey breeding in captivity is still new.

Some improvements may be expected from segregation of monkeys, conditioning of monkeys, long-term quarantine, and perhaps, freezing of pretested cultures (also through laminar flow to be discussed later by Dr. Coriell).

The second part of the program dealt with antigens in cultures derived from the tissue source rather than from viruses. This was presented in a comprehensive review of Dr. Coriell.

The vaccines now used contain many adventitious nonviral antigens because they were produced before the development of the more modern, sophisticated methods of analysis. Dr. Coriell cited one example where vaccines were believed to contain no host antigen, yet were shown to contain egg material, egg antigen, when tested by the passive transfer technique. Blood group A substance has been found in influenza vaccines. Dr. Coriell pointed out that it is desirable to get rid of these antigens. Fortunately, with the switch from egg to tissue culture, the purity of vaccines has been enhanced. For example, measles vaccines made in chick embryo fibroblasts *in vitro* do not give reactions in egg-sensitive individuals.

But a new, disturbing problem has emerged recently: the reaction in people given killed measles vaccine to subsequent natural infection or immunization with live measles virus. The mechanism of this reaction remains unknown. It is apparently not associated with cellular allergies because it has occurred among children given killed monkey vaccine and subsequently chick embryo vaccine or infected naturally with the virus.

I would like to call attention to the virtual absence of reactions among millions of people who have received polio vaccine prepared in tissue culture.

Serum antigens have been eliminated from vaccines by virtue of actual washing away of medium before infection and subsequent treatment with trypsin, which digests some of the extraneous protein. The only major problem remaining until recently has been penicillin, which has given reactions, but this is being controlled by the substitution of other antibiotics.

In general, Dr. Coriell and also a discussant, Dr. Franks, stressed the extremely low risk of reactions that can be anticipated or can be traced to tissue antigens in vaccines.

Neoplastic Properties of Animal Cell Lines—Summary

THELMA B. DUNN, *Laboratory of Pathology, National Cancer Institute,*[1] *Bethesda, Maryland 20014*

I will be very brief, because Dr. Furth has some important thoughts to convey regarding the influence of the host in altering the possibilities of neoplastic cell transformation. I have therefore selected particular points from some of the presentations—often because I have a personal bias for or interest in these points. If I make any incorrect statements, I hope to be corrected by the speakers.

Dr. Foley said there are no *in vitro* criteria for the recognition of a neoplastic transformation. Some oncogenic lines may be diploid and some heteroploid. Some heteroploid lines may fail to produce tumors. No biochemical markers have been recognized, and no reliable antigenic distinctions can be made.

Dr. Dawe said that the oncogenic potency of cultures may vary during continuous transfer. He mentioned several lines from which, after a period in culture, tumors failed to develop although originally the cells were obviously neoplastic. He called attention to certain similarities between virulence of microorganisms and neoplastic potentialities of cells.

Dr. Sandberg emphasized that no two tumors and no two culture lines are precisely alike. Many changes observed in neoplastic tissues are secondary and not primary.

This takes one back to the early days of experimental cancer research when studies on the transplantation of tumors were first made in mice. Bashford stated that there were no morphologic features characteristic of neoplasia and that the morphologic changes observed were merely concomitant with the neoplastic state.

Dr. Sandberg stated that cell lines from the same origin may very well differ, and in the discussion, the need for more study of embryonic tissues was emphasized.

Dr. Yerganian described a particularly interesting system involving

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Chinese hamster cells and noted that the cultured cells were good sources for continuous diploid lines because they were remarkably stable.

Dr. Defendi said that the possibility should not be forgotten that some oncogenic cells may arise very early during *in vitro* growth and that primary cultures as opposed to continuous cultures carried the least risk.

Dr. Todaro reported his experience with culture lines derived from human beings. He noted that cells obtained from patients with Fanconi's anemia underwent chromosomal aberrations readily. He found tissues derived from young individuals were more stable than those from older individuals, and that the changes in earlier passages were less easily detected than those in later passages.

It would thus appear that in obtaining human cell cultures you would do well to select young, healthy individuals in preference to others.

Dr. Kruse described continuous perfusion methods of culture and suggested that these methods might be useful in obtaining heavier yields of virus per culture vessel. With perfusion methods, contact inhibition was not manifested, and cells could be grown in multilayers. This led to a debate on the importance of contact inhibition as a criterion for neoplasia.

I had seen Dr. Sanford's paper in advance, and I am also acquainted with the work in this laboratory. This work has generally been carried out, as you know, with mouse cells. The statement was made that the mouse cells were the ones most liable to undergo neoplastic change. That may be true, but it is also true that they are the ones that have offered the best opportunity to make real tests for neoplastic changes, since the cells can be returned to the inbred mouse strain from which they came.

Dr. Sanford pointed to the recent work of Dr. Evans which indicated that fetal calf serum in the culture medium will maintain a normal nonneoplastic cell line for longer periods than a medium containing horse serum. This raises the question whether there is some inhibiting substance in the calf serum or some substance within the horse serum that causes the neoplastic change.

Dr. Barski discussed the cultivation of neoplastic cells and non-neoplastic cells together and observed that the resulting hybrids can be highly neoplastic.

Dr. Moore described her work with tissue culture cells transplanted to human beings who were terminal cancer patients. Very large doses of the cells were required to produce growths. The controls were young, adult, healthy individuals, and no growth resulted in them.

Dr. Macpherson said that, if live cultures were used, there was little danger of transferring live cells that would grow in recipients of vaccines. Choice of species from which cultures are obtained is important. *Mycoplasma* contamination was a hazard and was especially frequent in horse serum.

Dr. Stevenson emphasized again that not all heteroploid cells are neoplastic.

Dr. Toolan discussed heterotransplantation to the hamster's cheek pouch as a means of testing for neoplasia. Growth of neoplastic tissue was obtained. None was obtained with normal tissue, and there was limited growth with embryonic tissue.

As a conclusion to these papers, I think it was generally agreed that the transfer and growth of cells carried in a vaccine were less serious hazards than the possible transfer of a virus. Also it was agreed that better methods for tissue culture should be developed and more information obtained along the lines indicated during this session.

Neoplastic Properties of Animal Cell Lines—Summary

JACOB FURTH, *Department of Pathology, College of Physicians and Surgeons, Columbia University, New York, New York 10032*

SESSION III gave ample documentation of the spectrum of transformation of normal cells in culture. The speakers and discussors agree that differentiation between neoplastic and non-neoplastic transformation cannot be made on morphological grounds. Diploid cells are usually not neoplastic. Heteroploid cells are often neoplastic, but perhaps even more often, non-neoplastic. Autonomous cancer cells are usually heteroploid, but can be diploid. Chromosomal markers of neoplasia rarely exist.

These observations are best explained by equating transformations with genome changes, *i.e.*, mutations, and by considering neoplasia as a special kind of transformation. The alternate supposition, that transformation is a disturbance in differentiation, contradicts the fact that differentiation is not associated with chromosomal changes.

Transplantation assays are the only means of judging neoplastic transformation, but this technique has marked limitations not only in isologous (syngeneic) hosts, but also in autologous hosts. Certain transplantable tumors can become nontransplantable during passages. This has been interpreted by supposing that this line, neoplastic at first, has become non-neoplastic. However, the opposite, *i.e.*, progression, is the rule. During subgenerations *in vitro*, as well as *in vivo*, further transformations can change a cell line, originally nonantigenic, into a line antigenic in the strain of origin, and thus eliminate it by immunosurveillance in the host in which it is grafted, while *in vitro* survival of this line is decided by its competitive advantage or disadvantage in the tissue culture media.

Little was said about the causes of cell transformation and of the underlying basic events. Transformations, as already stated, are best conceived as due to changes in the genetic code. They can occur spontaneously as a consequence of error in copying DNA in the absence of a carcinogen. The chances of such errors are greatly enhanced in cultures by driving the cells to replication, as occurs *in vivo* by application of hormones.

Carcinogens are best conceived as belonging to agents that are capable of changing the cell code. Most common of these are the omnipresent ionizing radiations, certain chemicals, and, perhaps most important from a standpoint of vaccine production, certain viruses.

Neoplastic viruses can transform cells by several mechanisms, of which the most important appears to be the complexing of viral NA with the cell DNA. It is remarkable that *in vitro* neoplastic cells can arise within weeks, if not in days, while *in vivo* the similar transformation requires several weeks or months. This difference has not been thoroughly analyzed by bioassays in properly conditioned hosts.

The basic events *in vitro* are not different from those *in vivo* with respect to transformation in general and neoplastic transformation in particular.

Neoplasia is a relative term. Many neoplasms are not autonomous but are conditioned by certain host changes, and the basic alteration can reside entirely in the host. If the change resides in the cell, it can be subtle, not recognizable by chromosomal modifications.

In cell cultures, the homeostatic regulators, coming from distant cells, are absent, and the cells proliferate frantically as they do in the body, when they are uninhibited. Because of homeostatic mechanisms, the degree of stimulation *in vivo* is either not as intense and not as lasting as in cells *in vitro* growing in consecutive passages uninhibited by such control mechanisms.

The homeostatic immunologic surveillance system being absent *in vitro*, the mutant heteroploid cells can pile up, both neoplastic and non-neoplastic, while in the host most of them are eliminated or restrained. A more thorough systematic analysis of these two types of cell lines utilizing hosts with induced lack of immunocompetence is yet to be done.

Thus, it seems that cell cultures speed up and exaggerate events which can occur *in vivo*, and that much can be learned by analysis in depth of the causes and character of cell transformation in cell cultures that are basic in oncology. This analysis may be of some value from the standpoint of virus vaccine production. Conversely, the latter discipline, rendered possible by cancer-oriented tissue culturists, may contribute to solving problems of cancer, notably in the area of viral causation of neoplasia and the possible control of some cancers by vaccines.

Viral Oncogenicity and Virus Vaccines—Summary

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Dr. Huebner pointed out that the list of known oncogenic animal viruses is getting longer each year and the number of species naturally infected with demonstrable tumor viruses is increasing. These viruses, capable of producing tumors in experimental animals, include representatives of many of the major classes of known animal viruses, and with the demonstration of the oncogenicity of adenoviruses they now include agents which cause natural acute infections of man. The RNA viruses probably represent the more troublesome group, since they appear to be more ubiquitous in nature and are present in a wide variety of species. Although to date no viruses have been demonstrated to be capable of causing cancer in man, this must be considered a likely possibility. The crossing of divergent species lines, such as the production of sarcomas in subhuman primates by RSV, an avian virus, as discussed by Dr. Deinhardt, lends further evidence toward the potential danger to man.

The possibility of contamination of cell cultures with indigenous viruses, including the tumor viruses, has been recognized since the early days of tissue culture. Recently, however, the increasing sophistication of virological techniques has demonstrated different and more obscure interrelationships between viruses and between the virus and its host cell. Thus, as discussed by Dr. Melnick and Dr. Vogt, the helper virus situation in avian and murine leukoses makes it possible for the presence of one virus to help the replication of a different, but related one. The genome of one virus then can be produced in a particle which has the antigenic coat of the other, probably by phenotypic mixing. The genomes of two different and unrelated viruses, such as adenovirus and SV40 tumor virus, may be associated in such a way that the capsid is that of the adenovirus and the genetic materials a functional part of each of the two, as explained by Dr. Rapp. There are also examples of completely dependent animal viruses, such as the adeno-associated viruses discussed by Dr. Smith and Dr. Rapp. Here the virus can replicate only in a cell supporting the replication of an adenovirus. These complex and obscure interrelationships can no longer be considered as laboratory artifacts, since the helper virus situation with avian leukosis agents and the adeno-associated virus dependency have been demonstrated under conditions of natural infection.

An interesting discussion of the terms of "latency" and "defectiveness" of viruses brought out the fact that, frequently, these are relative terms, depending on which system is used under which conditions.

It was apparent during the session that a number of effective techniques are already available for demonstrating the presence of contaminating known viruses. Direct isolation of the infectious virus is still the most commonly used method and has the advantage of biological amplification as the agent multiplies in the test system. After such multiplication, other techniques, such as electron microscopy and immunological methods, can be applied. It was emphasized that long-term observation and even subculture of the test system can be important, although this increases the chance of accidental contamination from the environment. When isolation techniques are used on the final virus vaccine product, antiserum to the vaccine virus must be used in the case of live virus vaccines. Such antiserum should be produced against highly purified virus antigen for fear it might also contain antibodies against some of the possible contaminants.

Immunological methods were discussed by Dr. Rightsel who pointed out the greater sensitivity of fluorescent-antibody (FA) staining over complement fixation (CF). The advantage of the serological methods is that they can demonstrate specific viral antigens even in the absence of whole infectious virus. Furthermore, they may be used to demonstrate the presence of virus-specific antigens other than coat proteins. The production of nonstructural T antigen specific for SV40 virus, for instance, is the only evidence for the SV40 genetic materials in the adenovirus-SV40 hybrid particles. The group antigens common to all avian leukoses or to murine leukemias demonstrable by serological methods offer a special advantage when one is looking for contaminating viruses.

Dr. Green showed us the elegant molecular biological techniques that can be used to demonstrate the presence of a masked or integrated viral DNA in cells oncogenically transformed by certain tumor viruses. Cellular messenger RNA will hybridize with the DNA of purified virus, as will also the DNA from a cell supporting replication of the virus. Dr. Axelrod, however, pointed out that for these techniques large amounts of highly purified virus are required so they are limited in their application.

The fusion of an SV40-transformed cell into a cell like the green monkey kidney cell, which is susceptible to lytic infection, can activate the production of infectious virus. This can be enhanced by use of inactivated Sendai virus to increase the efficiency of cell fusion and represents a possible means of looking for virus integrated into a transformed cell.

When we consider the ways to demonstrate the presence of a new contaminating virus not previously known, there are fewer methods beyond that of virus isolation. Electron microscopy is limited because many virus particles are required before they can be seen, but the physical methods presented by Dr. Anderson might produce the necessary concentration. The new methods of using density gradients reviewed by Dr. Anderson are not only applicable to purification of the vaccine virus, but might give evidence of contaminants of different densities.

In the search for unknown agents, an FA-staining technique was suggested and some evidence of actual use was presented. γ-Globulin from pooled serum of adult animals of the species supplying cells for vaccine production cultures would be used, the assumption being that the globulin might contain antibodies against all the usual indigenous viruses of that species. Where noninbred species are used, however, one would have to rule out positive reactions being due to isoantigen-antibody interaction.

The demonstration of interference to challenge with a known virus or of interferon in the cell culture to be used for vaccine production was evaluated by Dr. Henle. Although a valuable tool, as already demonstrated in the case of the avian and murine leukoses, a negative test for interference or interferon does not rule out an indigenous virus and the presence of interferon does not necessarily mean the presence of such an agent.

It was pointed out that, even when a known virus is found contaminating a virus vaccine product, it can be removed in some instances by the application of physical or chemical methods. However, the aim in biologic production is for cell cultures originally free of contaminants.

Finally, in considering the hazard to vaccinees who receive virus vaccines possibly containing extraneous tumor viruses, it was felt that certain host elements represent safety factors. In general, large doses of viruses are required to produce tumors in experimental systems; this is especially true when immunologically mature animals are used and when species lines are crossed. If there are viruses tumorigenic for man, they are likely to be ubiquitous and some individual factor such as immunologic competence might determine which infected person will develop a gross tumor. This certainly is true in some animal systems, such as natural infection of mice with polyoma virus. Fortunately newborn humans are immunologically mature compared to the highly tumor-susceptible newborn hamster.

Experience With Vaccines—Summary

Hilary Koprowski, *M.D., and* George Gee Jackson, *M.D., University of Illinois, College of Medicine, Chicago, Illinois 60612, and Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104*

DURING this session, encouragement was obtained for further development of the program for surveillance of late effects among population receiving viral vaccines. This has been accomplished by record-linkage to identify clusters of specific abnormalities and permit cohort analysis. Utilization of these techniques for as many as 10,000–15,000 doses of virus vaccines, some of which subsequently became known to have unwanted and unexpected agents, has failed to show a dose relation between presence of SV40 and leukemia. In addition, no excess in mortality figures or increase in incidence of any specific disease, including malignancy, was observed in the population receiving these vaccines. Extending the time of observation to 6–8 years post vaccination will increase the validity of this surveillance, and the availability of several types of vaccine in the same program may make evaluation of a single condition more certain.

In developing this program, some attention was paid to techniques which may show benefit of vaccines other than prevention of the acute illness, such as decrease in the incidence of mental retardation or prevention of chronic diseases.

For the program to be even of minimum effectiveness, physicians' death certificates must improve vastly in accuracy, and a national death index must be created to determine whether vaccinated individuals are still living.

It is hoped that this program will be conducted on an international level, since countries other than the United States may have already started it and may make valid contributions to its success.

In view of the enormous population of animals (over 7 billion doses of culture vaccine being used) vaccinated with viral vaccines—produced in cells of homologous and heterologous origin and representing either primary cultures, diploid lines, or permanent lines, including frankly oncogenic lines—adequate surveillance of the vaccinated animal population may provide data of tremendous value. It is recognized, however, that because of the brief longevity of some species, this surveillance program may be difficult to apply to some species. This should not preclude establishment of a surveillance program for domestic animals on the same basis as for human populations.

No definite criteria can be established at this time of suitability of heteroploid lines for virus vaccine; however, heteroploidy of a cell line cannot be always directly related to malignancy, and frankly malignant cells treated as for virus vaccine production did not increase frequency of tumors in laboratory animals. Greater complacency may have been expressed, however, for cell lines of known character than for primary cultures of the same species.

Vaccines produced in human diploid cell lines (HDCL) for at least 4 viral agents have been given to 700,000–800,000 people by either enteric or parental route, with no recognized adverse reactions after more than 3 years' observation. Within this time limitation of evaluation HDCL should now be considered suitable for production of viral vaccines.

Feelings were expressed clearly that multiple types of cell culture, either of the same or of different species, should be considered for vaccine production. It was also stated clearly that results of surveillance of one or another product will not provide the answer soon enough to be a criterion for acceptability of one type of cell culture rather than another. Reasonable judgment must be made on the basis of currently available data and analogy to other products. The system with fewest unknowns would be preferable.

Feelings were expressed, which we strongly endorse, that the ultimate aim of viral vaccine development should be the antigen itself without any additional by-product. The time for that will come. In the meantime, however, we should use a multiple approach with regard to cells used for vaccine production.

Ultimately, *homo futurus*, who may not necessarily remain *sapiens*, will be vaccinated and hopefully immunized with a viral antigen made in a cell-free test tube system and containing no genetic information. Our descendent will always carry a small bottle for spray or injection containing double-stranded RNA for production of his very own interferon. I am sure, equipped in such a way, he can live happily not only on earth but also on Venus, nonfiguratively speaking.

GENERAL DISCUSSION

Dr. A. J. Kniazeff: I would like to emphasize the probable usefulness, in the study of diploid cell lines or established cell lines for vaccine production, of scrutinizing the large animal population that we do have.

Maybe most people are not familiar with the fact that the veterinary profession has established a definite, recognized nomenclature for all the diseases of animals, and that this nomenclature has been computerized. It is already in use in several of the veterinary school diagnostic laboratories and clinical departments where all of the information that has been gathered on diseased animals is fed into computers. I understand that in Europe there are several veterinary schools also interested in utilizing this system.

The question was asked before: How long do the animals live? Most of our large domestic production animals do not live very long. But from my experience as a practitioner I know that cats and dogs are kept for a very long time. In fact, they usually get more attention in the family than children do.

Another advantage of following the animal population is that, let's say in the United States, most of the States require that a record be kept of vaccination, particularly for rabies. In many States although not necessarily required, often the data on distemper and hepatitis vaccination are also included. So it would not be a large task to accumulate data on a statistical basis for a large number of animals, which would include such information as the type of vaccines the animals were given, at what time, and how many times, and also perhaps include such things as development of tumors in animals not vaccinated or in those vaccinated with killed vaccines versus vaccines prepared in diploid cell lines and also produced in established cell lines.

Dr. P. D. Cooper: I'd like to make two general points. The first one is about pressures. We have heard a lot in this Conference about the risks of the vaccines being matched against the need, and we also have heard rather a lot about the risks of vaccines. We haven't heard much about the actual need for vaccines. I think that, if a body were to sit down and evaluate the risks involved, it might be useful to have a systematic evaluation of the potential needs too. There are several virus diseases that need a vaccine and dominant among these is influenza.

It's now 10 years since the Asian flu outbreak. I would very much like to be contradicted on this, but my impression is that our capacity for influenza vaccine production is not much better now than it was 10 years ago. I hope I am wrong; but if we were to get another outbreak of the 1918 sort, I think we would be in trouble, and I think that this is a much more real risk than the risk of, shall we say, using diploid cell lines.

The second general point I'd like to make is about the large-scale cultivation of diploid cells. I was talking earlier about large-scale cultivation of continuous cell lines because they can be cultivated easily in a chemostat system. However, continuous production of these cells for vaccine manufacture would be microbiologically unsound because one should frequently return to a master seed. Thus there is no advantage, from the production viewpoint, for continuous line cells over limited passage lines such as the diploid cell lines.

The feasibility of growing diploid cells on the surface of little beads of Sephadex in suspension, as reported recently by Van Wezel [*Nature* (*London*) 216: 64, 1967], would give the advantages of bulk suspension culture for cells like the diploid cells, which can be grown only in monolayers at present.

Laminar Flow Systems for Sterile Work

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I want to make just two points about contamination: 1) It is a continuing and serious problem in the use of tissue cultures and 2) there are new techniques which may reduce the amount of contamination if applied in all these procedures we have discussed in the last 3 days.

Dr. Barile reported that 40–92% of heteroploid, serially passed cell lines were contaminated with *Mycoplasma*. If *Mycoplasma* can get into 40–92% of the cultures, then obviously other organisms can also get into cell cultures.

We know without documentation that bacterial contamination of cell cultures is very frequent in all laboratories.

The following list shows the human polyploid epithelial-like cell lines which contain the G6PD isoenzyme variant type A.

Three laboratories have tested these cell cultures: one laboratory using starch gel, another acrylimide gel, and the third a paper chromatography technique. All tests of the 41 cell lines agreed 100%, and 40 of the 41 cell

Human Cell Cultures That Contain G6PD Isoenzyme Type A[1]

1. HeLa
2. HeLa 229
3. HEp–2
4. Intestine 407
5. Minn EE
6. J–111
7. WISH
8. Girardi heart
9. Chang liver
10. Detroit-6
11. Detroit-6, clone 12
12. AV3
13. FL
14. KB
15. LI32
16. TuWi
17. Wong-Kilbourne conjunctive
18. Detroit-98
19. Detroit-98S
20. Detroit-98/AG
21. Detroit-98/AH–2
22. Detroit-98/AH–R
23. NCTC 2544
24. NCTC 3075
25. HeLa S3
26. HeLa 8/8
27. RH
28. Det-B16
29. Det-30A
30. Det-56
31. Det-78
32. Detroit-B17
33. Detroit-52
34. Det-96
35. Detroit-196
36. Det-110B
37. Det-34
38. Det-173
39. Detroit-501
40. Detroit-32

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[1] Analyzed by Institute for Medical Research, Camden, N.J.; Child Research Center, Detroit, Mich.; and American Type Culture Collection, Washington, D.C.

lines contained the A variant of G6PD which is found in HeLa cells. This suggests, but does not clearly prove, that most heteroploid human cell lines have become contaminated with HeLa cells. This interpretation would be more secure if it were supported by more data. Other isoenzymes are under study to settle this point, and it is anticipated that identification of different cell lines within species can be accomplished with a battery of isoenzymes.

The LDH enzyme is a rapid test for identity of certain cells; for example, three cell lines we tested during the past year showed the LDH pattern of L cells instead of human cells.

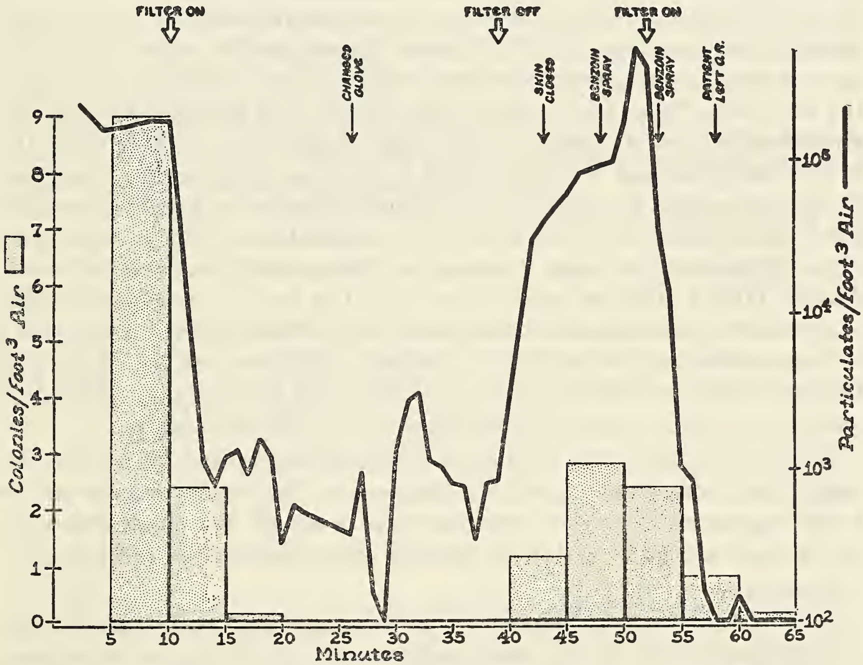
These observations help to underscore the need for better techniques to prevent contamination. I want to briefly describe new techniques which provide this capability, *i.e.*, filtration of air through high-efficiency filters to remove particles larger than 0.3 μ and direction of the filtered air to bathe a work bench, room, or hood. These filters are called HEPA filters or absolute filters. The HEPA means "high-efficiency particulate air" filter. They are made of cellulose acetate and, to meet government specifications, must remove 99.97% of all particles larger than 0.3 μ. Air comes from the filter at a speed of around 100 feet per minute. If the ceiling or one wall is made of a solid bank of filters and the floor or opposite wall is perforated for escape or return of air, we have a continuous flow of dust-free air, or so-called laminar flow principle developed by Dr. Whitfield at Sandia Corporation.

In a hood or room, the air flow can be directed from side to side or from back to front. Another procedure is to place the filters in a remote cabinet and duct the filtered air to the top or side of a room or hood with return flow by another duct. This provides various air-flow rates, portability, and flexibility of installation in existing buildings.

These techniques have been in use for several years in the electronics industry, particularly in the space program, for assembly of electronic gear that goes into the spacecraft. It was found by trial and error that assembly made in a dust-free environment prolongs the life of delicate instruments. It has been estimated that electronic systems assembled in a dust-free environment give up to 50-fold greater trouble-free performance. Unfiltered air contains many dust particles which tend to settle and aggregate and eventually cause malfunction of instruments with very fine tolerance. Tests have shown that the filters are also highly efficient in removal of airborne bacteria.

An example of the efficiency of the system is shown in an operating room equipped with vertical-flow filtered air at the rate of 19 feet per minute. Air was tested with a Reynier's sampler at the rate of 1 cubic foot per minute. Before the HEPA filters are activated, the bacterial counts vary from 5–10 per cubic foot. When the filters are turned on, the counts drop to zero within 1 minute. When the filters are turned off, the bacterial counts return promptly because the dust and bacteria swirl up from the floor. This same prompt effect has been observed in the laboratory, in hoods, and in the operating room.

Text-figure 1 shows one sequence from the operating room. Bacteria per cubic foot of air are plotted as solid blocks for each 5 minutes, and dust particles per cubic foot of air are plotted as a solid line. At the beginning of the record, with the filters off and 8 people in the operating room, there were about 9 bacteria per cubic foot of air. When the filters were turned on, the bacterial counts dropped to zero, returned when the filter was stopped, and again dropped to zero when the filters were restarted. Particulates larger than 0.3 μ were approximately 200,000 per cubic foot air at the beginning and dropped promptly to 100–1000 when the filters were in operation. Eight people in the operating room were generating bacteria and particles as they walked or moved during the operation, but the continuous stream of clean air carried these particles to the floor and out of the ambient air of the room.



TEXT-FIGURE 1.—Colonies and particles present during herniorrhaphy.

To measure in a different way the relative sterility of filtered air, we put 60 petri dishes for 4 hours in 2 locations: in the clean room equipped with vertical HEPA-filtered air and in two 4 × 5 foot transfer rooms. These are the rooms where we process all of the cell cultures without antibiotics for the cell bank and where we practically never observe contamination. After 4 hours' exposure, all petri dishes in the HEPA-filtered room were sterile and all 60 petri dishes in the transfer rooms were contaminated. Studies in the unmodified transfer rooms show that airborne bacteria increase when the technician is in the room and decrease when she steps out. After HEPA filters are installed, the same sampling techniques recover no bacteria with the technician in or out of the room.

I think these filters and air-flow techniques will help prevent contamination of cell cultures and vaccines prepared from cell cultures.

Finally I want to describe a laminar flow biohazard hood that we have developed to protect the technician as well as the microbiological procedure. For example, high concentrations of Schmidt-Ruppin Rous sarcoma virus may be infectious for man. Certainly Brucella, Q fever, and tuberculosis specimens should be confined to prevent exposure of personnel to infectious aerosols.

The hood is 4 feet wide and 24 inches deep with a 9-inch opening across the front for the operator's arms. HEPA filters pass sterile air in a vertical direction which exists through perforations at the front and back of the work surface. Ninety percent of the air is recirculated, 10% is discarded through another HEPA filter at the top, and an equal amount of air is drawn in at the front opening to create an air curtain which isolates the work area. Tests with smoke bombs in the hood demonstrate that the airstreams behave as predicted and that smoke does not leak from the hood during normal microbiological techniques. Tests with aerosols of *S. marcescens* and T3 coliphage generated inside the hood and just outside the front opening show less than 0.01% penetration of the air curtain in either direction. No viable bacteria or phage could be detected at the discard HEPA filter on top of the hood. The hood thus achieves better than 99.99% protection under the conditions of these tests. A front panel with portholes can be installed, if desired, and gloves can be attached to these portholes to achieve a class III hood. This panel is also installed to make an air-tight hood for sterilization with ethylene oxide.

Most of our tests have been done with the front panel off so that the work area is completely open for easy access. The results I gave on the percentage protection were with the front panel off. We think this hood is the best available and will prevent many laboratory infections of personnel.

A number of companies make other types of laminar-flow hoods that are satisfactory for routine tissue culture and microbiological techniques not hazardous to the technician. A hood in which the filtered air moves from back to front toward the operator is widely used in the space industry, and is perfectly satisfactory for routine tissue culture, media preparation, dispensing agar into Rodac plates, etc. I know Dr. Merchant has obtained one of the Edgeguard hoods and does tissue culture in it.

We installed our first hood in an animal room and demonstrated that we could do sterile work in this contaminated environment.

GENERAL DISCUSSION

Dr. D. J. Merchant: I might add that, as Dr. Coriell mentioned, we purchased one of these hoods recently to use in an area where we are doing fermenter work. The room was built just off our animal floor. It was designed originally for bacterial fermentation work and was never intended for tissue culture. We are having excellent success with the hood in this room. We couldn't possibly operate in the area otherwise.

Dr. M. R. Hilleman: Who changes the filter? That ought to be a real hazard.

Dr. L. Coriell: I don't think it's much of a hazard in practice. We put an ethylene oxide valve on the biohazard hood for people concerned about it. You can install the front panel, fill the hood with ethylene oxide, and leave it overnight to sterilize the hood and filter.

Dr. Hilleman: How long does one of those filters last before it's spent?

Dr. Coriell: Our oldest filter has been in use for almost 2 years and has not been changed yet. We have changed the pre-filters 3 times. I forgot to mention that a pre-filter of the type used in your furnace is inserted ahead of the HEPA filter. When the pre-filter gets filled with coarse dust, the air flow is reduced and this is registered on a magnahelic gauge. When the gauge shows the air is not moving fast enough, the pre-filter should be changed.

I understand that in the space industry HEPA filters frequently last for 2–3 years. Apparently the tiny particles that get down to the HEPA filter get dried up by all the air passing over them and they don't plug the final filter.

Dr. H. von Magnus: I understand the name of the filter is HEPA. What is the name of the cabinet?

Dr. Coriell: The biohazard hood I described is made by the J. T. Baker Co., Biddleford, Maine.

Dr. von Magnus: We have imported one from the United States—not of this type. The air comes out in front of the person sitting in front of the cabinet, and we have found it very practical for a number of purposes. But I was anxious to know whether air comes down and not out to the person.

I would also like to say that this particular cabinet, which we are very glad to have, has one very unpractical feature: The person sitting in front of it doesn't sit very comfortably. We have had to change it and lower it. I think that a physiotherapist should look at the whole setup because it's not very comfortable.

Dr. Brennan: Is my inference correct that you do not use a Bunsen burner in the work area?

Dr. Coriell: We do and we don't. It's almost impossible to change people from using a Bunsen burner when they have been using one for many years. So some of our workers use Bunsen burners in the hood. I tell them they don't have to and can demonstrate that they don't have to, but some continue using a Bunsen burner. The air doesn't move fast enough to interfere with using a Bunsen burner.

Dr. Brennan: Do you have a heat buildup in the system then? I notice you have 10% removal of air there at the top. Does that take care of the heat buildup?

Dr. Coriell: Yes, that is no problem with the hood. If you have a large room, such as a 12 × 12 clean room or the 14 × 18 room being installed, you should build in some air conditioning because heat builds up in the large room, especially in the operating room which contains 6–12 people in addition to heat from lamps and motors. But for these small hoods you don't have to worry about heat buildup.

Dr. Merchant: For those who wish to use horizontal laminar-flow hoods for non-infectious materials such as cell cultures the model, called the Edgeguard hood made by the J. T. Baker Company, has some advantages over others. If you put a beaker or other object in the hood in the horizontal-flow models, you tend to get turbulence behind the object, which can alter the flow and pull material into the hood. The Edgeguard has a vacuum around the face of the hood which pulls the air forward at the same time that it is being blown forward and so avoids this difficulty.

Virus Vaccines—Perspectives for the Future

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Research in the field of immunoprophylaxis is mainly empirical. If a vaccine is found to be useful, all is well. The questions how it works and why it is or is not effective are seldom asked. A more rational approach to the problem of immunoprophylaxis, including systematic studies of the basic mechanisms of protective immunity, is undoubtedly to be desired.

The reason why this aspect of the problem has been largely neglected is at least partly due to the fact that, in the virus field, the trend has been decidedly in favor of live attenuated vaccines. It is apparently taken for granted that such vaccines will produce a state of immunity, in all respects equivalent to that following natural infection, so there is no need to worry about the mechanisms.

However, the last few years have added much new information con-concerning the oncogenic properties of viruses, persistent infections, and slow-virus phenomena. We still know very little about the conditions under which these various manifestations of virus activity occur. In this situation, a certain caution in mass application of live virus vaccines would seem desirable. I am not thinking now of the possibility of an unintentional introduction of extraneous viruses. This may present a serious problem all of its own, but it is of technical nature and it can no doubt be solved. I am thinking of effects of the immunizing agent itself besides and beyond those we intend to produce and that we might be unable to control. The probability that side reactions, if such occur, will appear only after the passage of many years should give added warning to proceed slowly.

There are, of course, situations calling for immediate action and in which the taking of considerable risks is justified. But the hazards, both known and potential, should always be assessed and weighed against the benefits to be gained. It should also be remembered that side effects, tolerated in the initial stage of a vaccination campaign, may appear unacceptable to the lay public, once a disease has been brought under reasonable control. The psychological aspect of the problem of mass prophylaxis always has to be considered, and continuous attempts should be made to improve the methods used.

The ideal immunoprophylactic agent should produce protective immunity but elicit no other reactions. Thus, not only should directly or potentially harmful side effects be avoided but also those by themselves harmless immune reactions that are without protective significance, since by antigenic competition they might interfere with the desired responses. For these reasons, it is of interest to gain information on the exact nature of protective immunity. This may well vary from one virus disease to the next.

MECHANISMS OF PROTECTIVE IMMUNITY

Present evidence indicates that three different mechanisms may be instrumental in the establishment of protective immunity: (*a*) so-called pathotopic immunity, (*b*) virus neutralization by circulating antibodies, and (*c*) cell-associated immunity.

(*a*) Pathotopic immunity is localized to the portal of entry of the virus in the mucous membranes. Sometimes assumed to be an expression of a specific cellular resistance ("tissue immunity"), it seems in fact to be mediated by local production and release of Ig A antibodies. The site of production is probably in solitary plasma cells and lymph follicles in the mucous membranes themselves. Ig A antibodies are mainly found in secretions from the mucous membranes and certain glands, and only in small amounts in the blood. In the secretions they appear in a dimeric form, two 7S molecules being coupled together by the proteinaceous so-called transport piece; circulating Ig A is monomeric. Circulating antibodies, Ig G and Ig M, as well as monomeric Ig A, do appear in the secretions, filtering through the membrane but only in very low concentrations, less than 1% of that in the serum. Dimeric Ig A, on the other hand, is readily secreted, also after passive, parenteral administration. Apparently, formation of the dimeric form requires presence of the antigen in the mucous membranes, and coupling with the transport piece seems only to occur in *statu nascendi.*

The secretory Ig A antibodies apparently function by neutralizing the virus before it has made contact with its epithelial target cells. They provide the first line of defense which protects against implantation of virus and formation of primary foci of infection.

(*b*) Of the circulating antibodies, presumably only those with virus-neutralizing capacity have any protective effect, by interrupting or retarding extracellular dissemination of the virus. No antibodies can prevent "metastasis" of infection by means of migrating, infected lymphocytes or a direct spread of virus from cell to cell through temporary or permanent protoplasmic bridges. Ig G antibodies are probably more effective than Ig M for two reasons: their higher avidity and the longer duration of the

Ig G "memory." Circulating antibodies are mainly formed in spleen and lymph nodes; antigens which do not penetrate beyond the integuments seem not to induce production of circulating antibodies. For induction of antibody formation, certain critical antigen dose levels must be exceeded; the threshold for an Ig G response is significantly higher than that for Ig M.

(*c*) So far, little attention has been paid to the possible importance of cell-associated immunity. If in operation, it might be expected to manifest itself mainly as a graft reaction, directed not against the virus itself but against cells infected with, and immunologically transformed by, the virus. This kind of immunity seems to be most easily induced by live, antigen-carrying cells or by free antigen in adjuvants of the Freund type. It should also be pointed out that antibody-mediated and cell-associated immunity may act antagonistically, a phenomenon known as "enhancement."

PATHOGENESIS OF VIRUS INFECTIONS

By and large, virus diseases may be divided into three main groups as regards pathogenesis.

1) In true respiratory infections the primary focus is to be found in the ciliated epithelium of the respiratory tract. Infected cells are soon detached from the basal membrane; virus is thus released mainly on the surface of the mucosa and does not penetrate into underlying tissue. Dissemination of infection occurs almost exclusively on the surface of the mucous membranes, the secretions serving as a vehicle for the virus.

Apparently, a high concentration of antibodies in the secretions, *i.e.*, pathotopic immunity, is the only means by which such infections could be effectively controlled. If the surface antibody barrier is inadequate and virus breaks through, rapid multiplication and dissemination will follow before any antibody booster reaction can take effect. Circulating antibodies participate only insofar as they filter through into the secretions. Therefore, to provide significant protection they have to appear in very high concentrations in the blood.

2) A large number of viruses seem to use as portal of entry the epithelium covering the mucosal lymph follicles of the digestive tract, pharynx, and the small intestines. Often a primary focus of multiplication is found in underlying lymphatic tissue. Infected lymphocytes, traveling in lymph and blood and repopulating lymph nodes and spleen, may then set up a systemic infection of the whole lymphatic system as the next phase. A true generalization follows when virus is being released from the cells and appears free in the bloodstream.

Pathotopic immunity might apparently serve the same purpose in these cases as in that of the respiratory infections. Since Ig A is demonstrable intracellularly in the mucosal epithelium of the intestines, such antibodies may even be relatively more effective against alimentary than against respiratory infection. However, since high-level pathotopic immunity seems to be of relatively short duration, the most important defense against infections of this type is undoubtedly afforded by circulating antibodies neutralizing the virus as it is released from the primarily infected cells. Neutralizing antibodies in blood, lymph, and tissue fluid thus form a second line of defense which can be reinforced by a secondary response.

In vaccinia and smallpox, generalization by viremia does occur, but, in addition, a local spread of virus within the epidermis directly from cell to cell seems to take place. This process which may result in progressive necrosis is unaffected by antibodies but can apparently be arrested by sensitized immune lymphocytes. Presumably, this is achieved by a graft type of rejection of the infected cells whereby, on one hand, virus synthesis is interrupted and, on the other hand, virus already formed is made accessible to neutralization by antibodies.

Cell-associated immunity is definitely known to play an essential role in the pathogenesis of virus-induced tumors. To what extent it is produced and serves any function in nononcogenic virus infections other than variola-vaccinia is not known.

3) The arboviruses represent a third group of viruses that are introduced through direct, parenteral inoculation. The primary focus in these cases is presumably to be found in the endothelial lining of blood and lymph vessels. The virus seems to be released directly into the bloodstream, and the next step may well be a systemic infection of the vascular endothelium. Circulating, neutralizing antibodies apparently play a major role in the defense against such infections.

INACTIVATED VACCINES

Apparently then, attention should be paid to the individual immunity pattern of each disease and the knowledge thus gained should serve as a basis for the development of immunoprophylactic procedures.

As already pointed out, administration of live attenuated virus by the *natural* route of infection might be expected to induce all those reactions essential for protection against infections with virulent viruses, and production of live vaccine might, therefore, be considered to be a natural solution to all our problems. In my opinion, however, live vaccines should be resorted to only when adequate protection cannot be obtained by any other means. The idea of warding off one infection by substituting another is not particularly appealing. The observations on the behavior of oncogenic viruses provided the first factual basis for a prudent attitude toward the use of live vaccines. The more recent findings concerning slow viruses and virus-induced immunopathies have made this even more justified.

Immunologically mature mice, when infected with lymphocytic choriomeningitis virus, react violently. The disease may be fatal; if not, complete recuperation seems to follow. In immature animals, on the other hand, infection seems to be completely inapparent. However, it persists for life and, according to a recent report, mice thus latently infected succumb later to a special kind of nephropathy. Whether this is a pattern of more general applicability is, of course, not yet known. However, the possibility that attenuation of a virus might be associated with an increase in its capacity of setting up persistent infections and the consequences this might have would seem to merit particular attention.

Production of circulating neutralizing antibodies with the aid of inactivated vaccines will probably not present any great difficulties. Establishment of true pathotopic immunity might, however, prove to be technically not feasible. Passive immunization with homologous, dimeric Ig A antibodies is a theoretical possibility but is, for all practical purposes, out of the question. For protection against respiratory virus diseases the only alternative to a live vaccine would then be production of very high titers of circulating, neutralizing antibodies to provide for a sufficiently high concentration in the secretions.

The rather modest effect of vaccination against influenza indicates that current vaccines and administration schedules do not come up to desirable standards. The present whole-virus vaccines contain many components that are not necessary for induction of the only relevant immune reaction, production of high titers of Ig G, neutralizing antibody. Some of these components are definitely not wanted—in the first place the pyrogenic material, which prevents administration of the large vaccine doses that are probably needed. An improvement of the quality of the vaccine appears to be within reach, since treatment with ether seems to eliminate the pyrogenic effect. A more adequate spacing of inoculations than is now practiced might also contribute to produce the high serum titers that are needed.

The use of certain inactivated measles and RS virus vaccines has resulted in appearance of unusual and sometimes aggravated responses to subsequent exposure to attenuated or wild viruses of the same types. These observations have naturally caused apprehension and seem to serve as a pretext for a condemnation of inactivated vaccines in general. The mechanism behind these adverse reactions is as yet obscure. One fact can be definitely stated, however. In each one of the cases observed, infection has taken place; *i.e.*, vaccination has failed to protect. In other words, the reactions have occurred only in connection with actual virus multiplication in the organism. It can be assumed that these complications are caused by *in situ* reactions between antibodies on one hand, and on the other, virus or more likely soluble antigens released from infected cells. Concerning measles virus vaccine, an improvement of the potency of the vaccine and a proper timing of booster injections will provide full protection and, therefore, presumably eliminate the risk of complications. Also purification of the antigen might serve the same purpose. In the case of RS virus it is less probable that improvement of the quality of the vaccine will prove to be a practical way out of the dilemma. Since maternal antibodies do not protect infants against infection, obviously circulating antibodies, if they afford any protection at all, do so only when present in extremely high concentrations.

Production of cell-associated immunity is supposedly of central importance in immunoprophylaxis against virus-induced tumors; it may be so in smallpox vaccination and possibly in other cases, such as in infections by members of the herpesvirus and adenovirus groups. The factual knowledge in this field is even scantier than in those previously discussed and the whole matter is open for speculation.

Since immunopathic manifestations often are attributable to cell-associated immunity, a brief discussion of virus-induced immunopathies may be justified in this connection. In the progressive Aleutian disease of mink, the entire lymphatic system seems to be thrown out of gear, whereas a nonprogressive manifestation such as postvaccinal encephalitis may represent an essentially normal response to a temporarily appearing foreign antigen that happens to be structurally related so some normal cellular antigen. Three different immunopathic mechanisms are thus conceivable: (*a*) virus-induced functional transformation of cells in the lymphatic system; (*b*) appearance of antigens, viral or cellular, capable of breaking through the tolerance barrier; and (*c*) antigen-antibody reactions in the tissues.

Of these (*a*) concerns only live vaccines and (*c*) was already considered. Reactions of type (*b*), *i.e.*, autoimmunity, could be elicited either by virus-induced cell surface antigens or by normal cell components, which are antigenically modified and released in connection with a virus-inflicted cell damage. In the former case, the appearance of the foreign antigen would be intrinsically connected with the process of virus replication and all enveloped viruses can indeed be expected to contain antigens of this type. In the latter case, the modified cell antigens would be released only if the virus causes significant cell damage; *i.e.*, autoimmune reactions would be correlated to the pathogenicity of the virus.

The antigens considered here are presumably of the transplantation type. Lipids probably enter as an essential constituent, being responsible for their immunogenic capacity rather than their antigenic specificity. Although production of autoimmunity cannot readily be achieved unless the antigen is incorporated in Freund's adjuvant, it would seem advisable to remove lipids from all inactivated vaccines whenever this is feasible.

The obvious need for an improved smallpox vaccine poses the problem of finding methods for production of cell-associated immunity with purified antigens. If establishment of cell-associated immunity is essential, we have at present no valid alternative to the live vaccine. True enough, a large number of different antigens have been isolated from vaccinia virus preparations, but we have not yet much information on which, if any, of these is responsible for production of protective immunity even if a suitable antigen were available.

However, the problem could be bypassed. Smallpox virus presumably enters through the mucous membranes of the nose and throat, and primary foci are probably set up in local lymph follicles. A viremic phase is followed by localization in the epidermis and formation of pocks. It seems reasonable to assume that neutralizing antibodies could prevent dissemination of the virus, although they have no effect on the development of skin lesions, once the virus has been introduced into cells of the epidermis. Thus neutralizing antibodies alone could provide full protection against the natural disease, while being relatively ineffective in preventing artificial cutaneous or percutaneous infection. This possibility seems well worth further study.

PURIFIED ANTIGENS

The line of reasoning now followed leads to the conclusion that purified virus antigens in most cases would provide the best solution to the problem of immunoprophylaxis. So far this possibility seems to have been tested in two cases only. It was found that a partially purified measles virus hemagglutinin indeed produced neutralizing antibodies in high titers and protection against natural infection. Similarly prepared influenza virus antigen induced production of antibodies without causing the febrile reactions common after administration of whole virus vaccine.

The advantages of purified antigen preparations would be several. In addition to those already discussed, the following might be considered. All the relevant antigens seem to be proteins. It would thus be theoretically possible to remove all nucleic acids without impairing the immunizing capacity. This would be a rational solution of the safety problems attending the presence of viral or foreign nucleic acids in vaccines. A number of purified antigens may also be combined to multipurpose vaccines which would simplify the vaccination calendar. As the number of "must" vaccines tends to increase, this is an important aspect.

However, some questions concerning inactivated vaccines still have to be conclusively answered. The common argument that such man-made immunity must be inferior to that produced by nature may carry some weight insofar as we have not yet learned how to produce a really efficient pathotopic immunity. It does not hold water, however, in such cases where the protective effect depends on circulating neutralizing antibody.

The question of the duration of immunity also has to be considered. Systematic follow-up studies of populations adequately vaccinated with high-potency, inactivated polio virus and measles virus vaccines do not yet cover sufficient periods of time to permit any final conclusions. However, the observations so far made have not justified the assumption of any principal differences in duration of immunity after killed and live vaccines.

If, after all, it should eventually turn out to be a difference in favor of the latter and this then had to be referred to a persistence of the virus in the organism, this would in my opinion be an argument against, not for, live vaccines.

Finally, there are situations in which the preparation of nucleic acid-free protein antigens might prove to be impossible. Poliovirus is a case in point. The empty capsids of this virus carry the H antigen which differs distinctly from the N antigen of the complete virus. Disintegration of the virion with release of the nucleic acid seems to transform the N into H antigen. Presumably the presence of the highly charged nucleic acid affects the gamma structure of the capsid protein and, if so, free protein with N configuration could not be obtained. Anti-H antibodies have no neutralizing capacity and are thus without protective value.

TISSUE CULTURES

Before concluding, I might be allowed to say a few words about the topic this Conference is supposed to deal with, *i.e.*, tissue cultures. The realization of the fact that apparently normal tissue may harbor and produce great quantities of potentially harmful viruses has been a cause for great concern, and this it should be. The recent incidents in German and Yugoslavian laboratories have further emphasized the hazards attending the use of primary tissue cultures for vaccine production. In my opinion this is a very serious problem, and a satisfactory solution has to be worked out. It goes without saying that the use of standardized cell lines, carefully monitored and with seed material of known quality kept in store, presents one solution, and perhaps the only one, to this problem. An increasing interest in this technique and expansion of its use in the years to come can be anticipated.

The ideal solution would be a cell-free system for production of virus or virus antigens. This may not be as utopian as it sounds. It has indeed been reported that immunologically typical tobacco mosaic virus protein can be synthesized in a system containing ribosomes, activated transfer RNA, and the proper messenger RNA. However, there is a long step from the demonstration of the theoretical possibility and the elaboration of practical techniques for mass production. Man will probably be on the moon long before such methods have been developed.

General Conclusions From the Conference

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I would like to close the Conference with a few words regarding what I feel are areas of general consensus.

There seems to be a real need for improved surveillance and for epidemiologic studies. I hope the word will go forward to those who are responsible for initiating such studies, and to those agencies which supply funds for such endeavors, because much useful information could be gained from more careful and more extensive efforts.

To state it very mildly, there is a recognition generally of the limitations of primary cultures and a desire to find alternate and backup systems.

It is the sense of the majority of those attending the Conference that we recognize the value of cell lines that can be obtained from selected tissues, that can be carefully controlled, that can be exhaustively studied and tested, and that retain normal characteristics. Though there is a hypothetical danger in the use of human cells, this appears remote, and in the present situation there seems to be little reason for reluctance to license vaccines made in the type of culture represented by the prototype WI–38.

A number of lines similar to WI–38 should be developed from human, nonhuman primate, and other animal sources so that, as more information is obtained and the need for a wider variety of vaccines is apparent, we will have an ample number and variety of systems with which to work. A number of participants pointed out that we should not have all our eggs in one basket.

No convincing evidence was brought forward to argue against the use of continuously propagated cell lines, either diploid or mixoploid, for virus vaccine production. Since many of these cell lines can be grown in suspension and can be handled with much greater facility than diploid cell lines of finite lifetime, they would be advantageous. However, it is considered very important to determine the mechanism(s) by which mixoploidy develops in cell cultures. Mixoploidy due to virus infection or to cross-contamination by cell lines would negate the use of these cells.

There is a general consensus that, in the long run, we should aim for purified antigens. This would be ideal, provided the purified antigen is an effective immunizing agent and gives sufficiently long-lasting protection. In this case the advantages for the use of continuously propagated cell lines are obvious.

It would be appropriate at this time to form a small group to formulate criteria for the use of cell lines. Such a committee could start with guidelines already suggested and with information available and determine what additions are required for the present situation and as other needs become apparent.

Comments

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I WOULD like to say that from the viewpoint of our Division, this Conference has been most valuable and helpful. I think it has given us a cross-section of where things stand as of today and has also provided the possibility for the people who have views which are extreme, at both ends of the system, to air these, and perhaps with this we can go forward.

The word "licensing" is frequently brought up. Systems are not licensed. There is no bar to the use of these cell systems for the production of vaccines used for experimental or clinical investigative purposes. This is going forward. But as of this date we have not had a single application for a product for injection produced in any such cell system. The Conference will certainly put us in a better position to meet our responsibilities if and when such license applications are received.

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[1] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Definitions of Terms Used in the Conference on "Cell Cultures for Virus Vaccine Production"[1, 2, 3]

THE field of Tissue Culture has developed very rapidly during recent years and tissue culture methodology has become a useful research tool in many branches of science. Consequently, scientists with various backgrounds and various aims have entered the field of Tissue Culture. As a result considerable controversy has developed over terminology, and this has hampered communications among scientists in the field. Therefore, in 1961 the Tissue Culture Association formed an Ad Hoc Committee on Nomenclature chaired by Dr. R. S. Chang, Harvard University; the members of the committee were: Dr. L. Hayflick, Wistar Institute; Dr. W. H. Murphy, University of Michigan; Dr. J. E. Shannon, American Type Culture Collection and Dr. G. Yerganian, Children's Cancer Research Foundation, Boston. This committee studied the problem very thoroughly and after three years of deliberation prepared a report on "Tissue Culture Terminology." This report accomplished a very important task; it consolidated thinking in some areas, and pinpointed areas where considerable disagreement still existed.

In 1964 a smaller committee was set up, consisting of Dr. V. J. Evans, National Cancer Institute, Bethesda; Dr. T. C. Hsu, M. D. Anderson Hospital, Houston and Dr. S. Federoff (Chairman), University of Saskatchewan. It was charged with the following duties: to further study the problem, to analyze the reasons for controversy on some terms, and to propose a generally acceptable terminology. This committee prepared a report on terminology and by means of thorough discussion with various groups of scientists, the report underwent five major revisions. The final, fifth draft of proposed usage of animal tissue culture terms was accepted by the Tissue Culture Association at its Annual Meeting on June 3, 1966 in San Francisco. It also received the approval of a number of workers in the field from abroad.

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[1] Reproduced with permission of the Tissue Culture Association.

[2] Previously published without the "Additional Definitions" in the *Journal of the National Cancer Institute,* vol. 38, pp. 607–611, 1967.

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The adopted text of the "Proposed Usage of Animal Tissue Culture Terms" follows.

Tissue Culture

Animal Tissue Culture is concerned with the study of cells, tissues and organs explanted from animals and maintained or grown *in vitro* for more than 24 hours. Dependent upon whether cells, tissues or organs are to be maintained or grown, two methodological approaches have been developed in the field of Tissue Culture.

(a) *Cell Culture.* This term is used to denote the growing of cells *in vitro* including the culture of single cells. In cell cultures the cells are no longer organized into tissues.

(b) *Tissue or Organ culture.* This term denotes the maintenance or growth of tissues,[4] organ primordia, or the whole or parts of an organ *in vitro* in a way that may allow differentiation and preservation of the architecture and/or function.

Explant

This term describes an excised fragment of a tissue or an organ used to initiate an *in vitro* culture.

Monolayer

The term refers to a single layer of cells growing on a surface.

Suspension culture

This term denotes a type of culture in which cells multiply while suspended in medium.

Primary culture

This term implies a culture started from cells, tissues or organs taken directly from organisms. It does not include cultures started from explants of tumors developed by injecting cultured cells into animals. Such cultures would be considered more properly as continuations of the injected cell line or strain. A primary culture may be regarded as such until it is subcultured for the first time. It then becomes a *cell line.*

Cell line

A "cell line" arises from a primary culture at the time of the first subculture. The term "cell line" implies that cultures from it consist of numerous lineages of the cells originally present in the primary culture. (*See* note following section on "substrain.")

Established cell line

A cell line may be said to have become "established" when it demonstrates the potential to be subcultured indefinitely *in vitro.*

Note.—With present knowledge about cell behavior *in vitro* it is really impossible to determine the point at which a culture has become "established," if the term "established" is taken to mean that the culture has acquired the ability to grow indefinitely. (Based only on experience with human fibroblast-like cells, a culture should exceed 70 total population doublings before it can be considered to be "established" or "permanent.") As new information becomes available, it will be necessary to determine for each species or tissue source the number of transplantations and conditions required to give rise to established cell lines.

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[4] The word "tissue" comes from a French word meaning "woven together" and a tissue in the biological sense is the woven mass of cells together with their intercellular substance. Histologists generally recognize four basic tissues: epithelial, connective, muscle and nervous tissues.

Cell strain

A "cell strain" can be derived either from a primary culture or a cell line by the selection or cloning of cells having specific properties or markers. The properties or markers must persist during subsequent cultivation. In describing a cell strain its specific feature should be defined; *e.g.* a cell strain with a certain marker chromosome, a cell strain which has acquired resistance to a certain virus, or a cell strain having a specific antigen, etc. (See note following section on "substrain.")

Substrain

A "substrain" can be derived from a strain by isolating a single cell or groups of cells having properties or markers not shared by all cells of the strain.

Note.—In everyday usage "cell line" and "cell strain" have sometimes been considered interchangeable. However, according to Roget's Thesaurus the terms are not synonymous. The word "line" is used most properly with the meaning of an *uninterrupted sequence.* In this context it is synonymous with such words as procession, series and succession. In relation to paternity, "line" is synonymous with tree, pedigree, descent and family. In relation to posterity, "line" is synonymous with straight descent. "Strain" is generally used with the meaning of *relationships of kindred.* In this context "strain" is synonymous with race, stock, clan and tribe. Therefore it is proposed that the term "cell line" in the field of Tissue Culture should imply an uninterrupted sequence of cell growth, whereas the term "cell strain" should imply a certain relationship of the cells; *i.e.* that they all have one or more common properties or markers for which these cells were specifically selected. (See "Cell strain.")

Clone

This term denotes a population of cells derived from a single cell by mitoses. A clone is not necessarily homogeneous and therefore the terms "clone" or "cloned" should not be used to indicate homogeneity in a cell population.

Cloned strain or line

This term denotes a strain or line descended directly from a clone. (See "Clone.")

Diploid cell line

This term denotes a cell line in which, arbitrarily, at least 75% of the cells have the same karyotype as the normal cells of the species from which the cells were originally obtained. It should be noted that a diploid chromosome number is not necessarily equivalent to the diploid karyotype,[5] because there are situations in which a cell may lose one type of chromosome and acquire another type of chromosome. Thus the karyotype of the cell has changed but the diploid number of chromosomes remains the same. Such cells should be referred to as "pseudo-diploid."

A description of a diploid cell line should include the actual numbers of cells examined, the percentage of diploid cells and their karyotype.

Heteroploid cell line

This term denotes a cell line having less than 75% of cells with diploid chromosome constitution. This term does not necessarily imply that the cells are malignant or that they are able to grow indefinitely *in vitro*. In describing a heteroploid cell line, in addition to the karyotype of the stem line the percentage of cells with such karyotype should be stated.

Note.—For more precise definition of the genetic state of the cells, according to A. Levan and A. Muntzing (Terminology of Chromosome Numbers. Portugaliae Acta Biologica, 7:1–16, 1963) the following terms should be used:

Haploid

1) The basic number of a polyploid series (symbol: x). Haploid in this meaning = monoploid.
2) The chromosome number of the haplophase, the gametic, reduced number (symbol: n).

Diploid, *triploid*, *tetraploid*, etc.

The double, triple, quadruple, etc., basic number (symbols: 2x, 3x, 4x, etc.).

Polyploid

General designation for multiples of the basic number, higher than diploid.

Heteroploid

1) In organisms with predominating diplophase: all chromosome numbers deviating from the normal chromosome number of the diplophase.
2) In organisms with predominating haplophase: all chromosome numbers deviating from the normal chromosome number of the haplophase.

Euploid

All exact multiples of x.

Aneuploid

All numbers deviating from x and from exact multiples of x.

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[5] The term "karyotype" has been defined as ". . . a systematized array of the chromosomes of a single cell prepared either by drawing or by photography, with the extension in meaning that the chromosomes of a single cell can typify the chromosomes of an individual or even a species. The term idiogram would then be reserved for the diagrammatic representation of a karyotype, which may be based on measurements of the chromosomes in several or many cells." A. Robinson, A Proposed Standard System of Nomenclature of Human Mitotic Chromosomes. JAMA 174 : 159, 1960.

Mixoploidy

The presence of more than one chromosome number[6] in a cellular population.

Endopolyploidy

The occurrence in a cellular population of polyploid cells, which have originated by endomitosis.

Subculture

This term denotes the transplantation of cells from one culture vessel to another.

Passage

This term is synonymous with "subculture," and can denote the passage of cells from one culture vessel to another. When this term is used, however, it must be made clear just what is being passed, because virologists use this term to denote the transfer of supernatant culture fluid rather than cells.

Subculture number

This term indicates the number of times cells have been subcultured; *i.e.*, transplanted from one culture vessel to another.

Subculture interval

This term denotes the interval between subsequent subcultures of cells. This term has no relationship to the term "cell generation time."

Cell generation time

This term denotes the interval between consecutive divisions of a cell. This interval can be best determined at present with the aid of cinematography. This term is not synonymous with "Population doubling time."

Population doubling time

This term is used when referring to an entire population of cells and indicates the interval in which, for example, 1×10^6 cells increase to 2×10^6 cells. This term is not synonymous with "Cell generation time."

Absolute plating efficiency

This term indicates the percentage of individual cells which give rise to colonies when inoculated into culture vessels. The total number of cells in the inoculum, type of culture vessel and the environmental conditions (medium, temperature, closed or open system, CO_2 atmosphere, etc.) should always be stated.

Relative plating efficiency

This term indicates the percentage of inoculated cells which give rise to colonies, relative to a control in which the absolute plating efficiency is arbitrarily set as 100%. The total number of cells in the inoculum, the environmental conditions, and the absolute plating efficiency of the control should always be stated.

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[6] The terms used to denote the genetic state of cells with respect to chromosomal content should not necessarily be applied to every 'count' obtained in the evaluation of a population, but should be reserved for characterizing cells or subpopulations only after consideration of all information from karyotyping and taking into account the limitations of artifacts and of sampling error.

Fibroblasts

Fibroblasts are cells of spindle or irregular shape, and as their name implies, are responsible for fiber formation. In cell cultures many other cell types are morphologically indistinguishable from fibroblasts. In organ and tissue cultures in which cell inter-relationships are preserved, fibroblasts may be identified using accepted histological criteria.

Fibroblast-like cells

In cell cultures various types of cells acquire similar morphology. Cells acquiring irregular or spindle shape are often referred to as "fibroblasts." However, the derivation of these cells or their potentialities, such as production of fibers, are usually not known. Therefore such cells are more properly called "fibroblast-like" cells.

Epithelial cells

This term refers to cells apposed to each other forming continuous mosaic-like sheets with very little intercellular substance, as seen *in vivo* or in tissue or organ culture.

Epithelial-like cells

In cell cultures epithelial cells may assume various shapes but tend to form sheets of closely adherent polygonal cells. However, the degree of cohesion of the cells can vary. When the only criterion for identification of such cells is their tendency to adhere to each other it is preferable to refer to the cells as "epithelial-like" cells.

Culture alteration

This term is used to indicate a persistent change in the properties or behavior of a culture; *e.g.*, altered morphology, chromosome constitution, virus susceptibility, nutritional requirements, proliferative capacity, malignant characters, etc. The term should always be qualified by a precise description of the change which has occurred in the culture. The term "cell transformation" should be reserved to mean changes induced in the cells by the introduction of new genetic material. The nature and source of the genetic material inducing the change should be specified.

Tumorigenicity

There are no criteria for determining the tumorigenicity of cells observed only *in vitro*. The tumorigenicity of cells can be determined by the behavior of the cells in animals. Whenever, therefore, tumorigenicity is referred to, the following information should be given:

1) Description of host injected (species, strain, sex, age, etc.).
2) Pretreatment, if any, of host.
3) Site of injection and number of cells injected.

4) Description of growth of the injected cells.
5) Histological description of tumor and relation of tumor to adjacent normal tissues.
6) Occurrence of metastases.

Toxicity

The term "toxicity," when used to describe an effect observed in cultures, is meaningful only when the effect itself is also described; *e.g.*, toxicity as evidenced by altered morphology of the cells, by failure of the cells to attach to surfaces, by changes in the rate of cell growth, cell death, etc.

APPENDIX

In reporting a new cell line the recommendations of the 1957 International Tissue Culture Meeting in Glasgow should be followed. It was recommended that authors should give the following information when first mentioning a cell line in the course of a publication:

1) Whether the tissue of origin was normal or neoplastic and, if neoplastic, whether benign or malignant;
2) Whether the tissue was adult or embryonic;
3) The animal species of origin;
4) The organ of origin;
5) The cell type (if known);
6) The designation of the line;
7) Whether the line has been cloned.

It was further suggested that the designation of the line should consist of a series of not more than four letters indicating the laboratory of origin, followed by a series of numbers indicating the line; *e.g.*, NCL 123.

In describing a cell line as much information about the cells as is known should be given. In characterizing a cell line it is advisable to follow the procedures developed by the Cell Culture Collection Committee and adopted by the American Type Culture Collection Cell Repository. The following information may be included in whole or in part:

1) History.
2) Subculture number.
3) Culture medium.
4) Growth characteristics.
5) Absolute plating efficiency.
6) Morphology.
7) Frequency of cells with various chromosome numbers in a culture.
8) Karyotype(s) characteristic of the stem line(s).
9) Whether sterility tests for mycoplasmas, bacteria and fungi have been done.

10) Whether the species of origin of the culture has been confirmed and the procedures by which this was done.
11) Virus susceptibility of a culture at a given subculture number.

A description of a *cell strain* should include the procedure of isolation, the specific properties of the cells in detail, the number of subcultures, and the length of time since isolation. It is also advisable to give a full description of the cell line or primary culture from which the cell strain was isolated.

In subsequent publications when reference is first made to a previously described cell line or strain, its species and tissue origin should also be included in addition to its accepted designation; *e.g.*, rabbit kidney cells, line XY 126.

S. Fedoroff, *Chairman*,
Committee on Terminology, Tissue Culture Association.

ADDITIONAL DEFINITIONS

Transformation

This term should be reserved for heritable changes. The author (speaker) should specify what is meant since this term has multiple usages. Transformation *is not* to be considered synonymous with "neoplastic alteration!"

Oncogenic

This term should be used to refer to an agent. It is recommended that it be specified as *oncogenic agent!*

Tumorigenic

May be understood to refer to a cell having the capacity to produce a tumor when placed in a suitable environment. *Tumorigenic cell* is recommended!

Neoplastic

An adjective referring to a new or abnormal growth or proliferation of cells. Usually refers to an abnormal cancerous proliferation.

Malignant

We urge that this term not be used.

Nononcogenic and *Nontumorigenic*

Should be used to imply under *accepted methods currently available.*

Terms used in describing particles seen in electron micrographs of oncogenic virus preparations and some apparently normal tissues.

Intracytoplasmic A particles—doughnut shaped, two shells 70 μ × 50 μ
Intracysternal A particles—doughnut shaped, two shells 70 μ × 30 μ

The following are extracytoplasmic:

Immature B particles—four distinct layers or shells with electron-lucent nucleoid
Mature B particles—eccentric electron-dense nucleoid
Immature C particles—three distinct layers or shells with electron-lucent nucleoid
Mature C particles—"target-like" particles with electron-dense nucleoid

VIRUS VACCINE PRODUCTION

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